Original Research

Calcitriol ameliorates damage in high-salt diet-induced hypertension: Evidence of communication with the gut–kidney axis

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Impact statement

Potential therapeutic strategies for high-salt diet-induced hypertension that target the gut microbiota are already being investigated. Vitamin D is well known for its role in calcium homeostasis, but a growing number of studies have focused on its new biological function in immune regulation. Therefore, we used rats with hypertension induced by a high-salt diet as the research model and examined their transcriptome and microbiome to conduct an overall analysis of the gut–kidney axis. We elucidated that diet and calcitriol play a key role in shaping the gut microbial communities and transcriptome expression. We observed the effects of salt and calcitriol on the composition of the intestinal flora and the immune system and explored the microbial patterns associated with this immune dysfunction. Our results present candidate pathways and genes to explore the relationship between high-salt diet, calcitriol, and hypertension on the gut–kidney axis.

Abstract

Several studies have established a link between high-salt diet, inflammation, and hypertension. Vitamin D supplementation has shown anti-inflammatory effects in many diseases; gut microbiota is also associated with a wide variety of cardiovascular diseases, but potential role of vitamin D and gut microbiota in high-salt diet-induced hypertension remains unclear. Therefore, we used rats with hypertension induced by a high-salt diet as the research object and analyzed the transcriptome of their tissues (kidney and colon) and gut microbiome to conduct an overall analysis of the gut–kidney axis. We aimed to confirm the effects of high salt and calcitriol on the gut–kidney immune system and the composition of the intestinal flora. We demonstrate that consumption of a high-salt diet results in hypertension and inflammation in the colon and kidney and alteration of gut microbiota composition and function. High-salt diet-induced hypertension was found to be associated with seven microbial taxa and mainly associated with reduced production of the protective short-chain fatty acid butyrate. Calcitriol can reduce colon and kidney inflammation, and there are gene expression changes consistent with restored intestinal barrier function. The protective effect of calcitriol may be mediated indirectly by immunological properties. Additionally, the molecular pathways of the gut microbiota-mediated blood pressure regulation may be related to circadian rhythm signals, which needs to be further investigated. An innovative association analysis of the microbiota may be a key strategy to understanding the association between gene patterns and host.

Keywords: Hypertension, calcitriol, differentially expressed genes, transcriptome, microbiome, inflammation

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Introduction

Hypertension is a growing healthcare burden and is a major risk factor for myocardial infarction, stroke, heart failure, and cardiovascular disease.[1] High-salt diet has been identified as an independent risk factor for hypertension. The reported mechanisms include kidney sodium retention, elevated blood volume, and increased peripheral vascular resistance.[2] However, the underlying mechanism of these pathological changes is not fully understood. Therefore, it is of great significance to

study the pathogenesis and explore effective drugs for treatment.

Multiple studies have confirmed a causal relationship between salt intake and high blood pressure (BP).[3] The intestinal mucosa is the primary absorption site for excessive salt. Excessive salt intake leads to changes in the gastrointestinal flora and functional disorders; recent studies have revealed the link to microbiota composition and hypertension development.[4] It was found that dietary sodium reduction increases circulating short-chain fatty acids (SCFAs), which are associated with decreased BP, suggesting that dietary sodium influences the gut microbiome.[5] In addition to its effect on BP, gut microbiota is associated with hypertensive target organ damage, for example, kidney and brain damage.[6,7] One study found that the changes in the intestinal microflora induced intestinal immunological gene expression and gut permeability and gut bacteria translocation into the kidney.[8] The gut and kidneys play an important role in regulating BP. However, these studies focused on the baseline microbial composition and changes in intestinal metabolic function. Little is known about the crosstalk between immune pathways and intestinal flora mediated by the gut–kidney axis under hypertension.

Vitamin D is well known for its role in calcium homeostasis, but a growing number of studies have focused on its new biological function in immune regulation. Studies have found that vitamin D restores the barrier functions and promotes intestinal innate immunity. An *in vitro* study found that calcitriol supplementation reduces the intestinal permeability of bacteria and restores tight junction protein expression.[9] Studies have confirmed that the differentiation and stability of Th1 and Th17 cell phenotypes is regulated by vitamin D.[10] Consistent with this, vitamin D has been shown to dampen the secretion of IL-17A and IL-17F in Th17 cells and ultimately improve the clinical manifestations of experimental autoimmune encephalomyelitis.[11] Furthermore, vitamin D inhibits Th1 cells, enhances the Th2 cell response, and inhibit the proliferation of B cells and their differentiation into antibody-secreting cells.[12] In the past decades, a large body of observational studies and experimental data indicated that vitamin D has a protective effect against the development of hypertension, and this is mostly attributed to the role of vitamin D signaling in the regulation of endothelial dysfunction.[13] However, the benefit of vitamin D on the immune system and intestinal flora in hypertension is not yet clear.

Potential therapeutic strategies for high-salt diet-induced hypertension that target the gut microbiota are already being investigated. However, the interaction among vitamin D, intestinal barrier function, microbiome, and immune response remains unclear. Therefore, we used rats with hypertension induced by a high-salt diet as the research model and examined their transcriptome and microbiome to conduct an overall analysis of the gut–kidney axis. We aimed to confirm the effects of salt and calcitriol on the gut–kidney immune system and composition of the intestinal flora and explore the microbial abundance patterns associated with in this immune dysfunction.

Materials and methods

Animals and treatments

Four-week-old Sprague–Dawley (SD) rats (male, n = 18; body weight = 157–198 g) were purchased from Shanghai Slac Laboratory Animal Co., Ltd. Animals were housed in a barrier environment with ambient temperature (22 ± 5°C) and 12/12 h light cycle. After one week of adaptation, the SD rats were randomly allocated into three groups (six per group): normal control (NC) group, high salt (HS) model group, and high salt diet plus calcitriol supplementation (CAL) group. The NC group rats were fed with a low-salt diet containing 0.3% NaCl; HS group rats were fed with a high-salt diet containing 8% NaCl; and the CAL group rats were fed with a high-salt diet containing 8% NaCl and 200 ng/kg calcitriol (Selleck Chemicals, State of Texas, USA). The dose of calcitriol was based on that used in previous studies,[14–16] which showed that the maximum dose of calcitriol was not more than 250 ng/kg per day did not cause serum calcium increases and vascular calcifications in rats with intact renal function.[17,18] Calcitriol supplementation with oral gavage was started after two months of high-salt diet and continued for one month. At the end of the experiment, the rats were injected with 50 mg/kg pentobarbital sodium. Arterial blood samples were collected by intubating the abdominal aorta, then sacrificed by exsanguination. The animal protocols in this study were supervised and approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Ethical permit number: SZY-201601007).

BP and physiological parameter measurement

BP was measured using a BP-98A monitor (Softron, Beijing, China) according to the operation instructions with the tail cuff method. The systolic BP of all rats was calculated as the average of three independent measurements. The body weight of the rats was recorded every four weeks. During the experiment, the intake of water and food in each group was recorded. Hematoxylin and eosin (HE) staining of the kidney and colon was performed using an HE staining Kit (Beyotime, Shanghai, China). Periodic Acid-Schiff (PAS) staining and Masson's trichrome staining of the kidney were performed using a PAS staining Kit (Beyotime, Shanghai, China) and a Masson's trichrome stain kit (Solarbio, Beijing, China). Subsequently, ImageJ software was used to determine the renal fibrosis. The colonic damage was scored according to the method by Luk *et al*.[19]

Biochemical analysis

Blood samples were centrifuged at 3000 r/min for 15 min at 4°C to separate the serums, and then stored at −80°C for the later assessment of renal function. Serum creatinine (Scr) and blood urea nitrogen (BUN) were measured according to the manufacturer's instructions of the biochemical kit (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China, A028-2–1; C011-2–1).

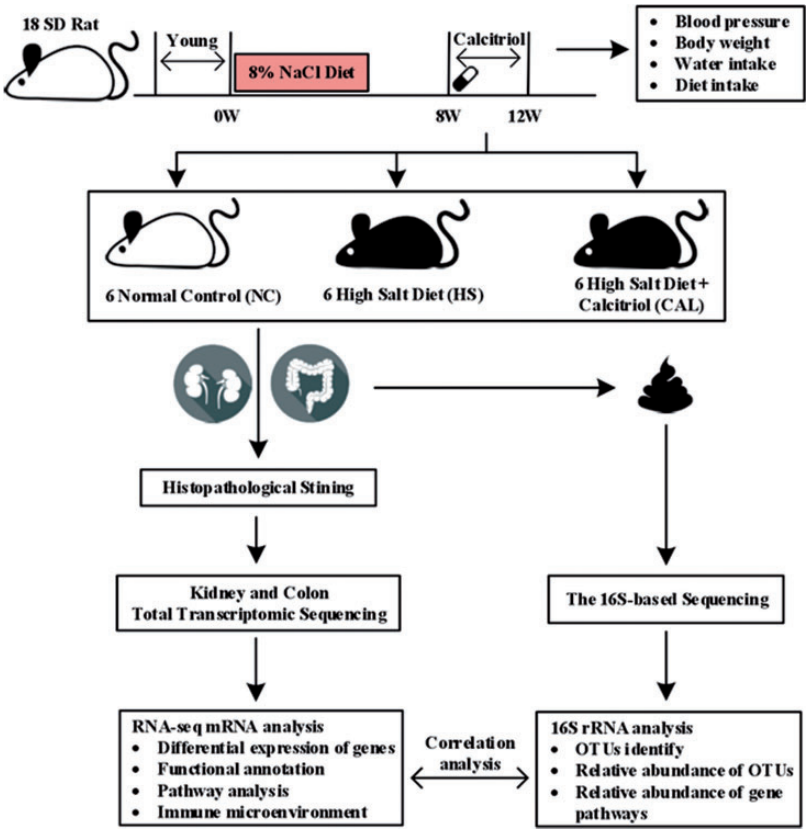

Figure 1. Experimental design and analytical procedures. SD rats were divided into a normal control (NC) group and a high-salt diet-induced hypertension model group. The model rats were further divided into two groups (n = 6 per group): the high salt (HS) diet group, the calcitriol (CAL) treatment group (200 ng/kg calcitriol per day). Histological analysis and caudal cuff blood pressure measurement validated the establishment of a hypertension model and the treatment effect. Transcriptomic sequencing and 16S-based sequencing were performed to unravel the therapeutic mechanisms. (A color version of this figure is available in the online journal.)

mRNA library preparation for sequencing

Total RNA of kidney cortex and colon was extracted using TRIzol reagent according to the manufacturer's instructions. cDNA libraries were prepared by TruePrep® DNA Library Prep Kit V2 for Illumina (Vazyme, Nanjing, China). Index-labeled libraries sized at 250–1000 bp fragment length were recovered by using VANTS® DNA Clean Beads (Vazyme, Nanjing, China). All libraries were quantified using a 2100 Bioanalyzer and pooled at a 1:1 ratio at 2 nM for HiSeq 75 bp single-end sequencing (Illumina, USA) and were further sequenced by Berry Genomics Co. Ltd (Berry, Beijing, China).

Transcriptome analysis

The quality of sequencing data was evaluated using FastQC (v0.11.9).[20] Adapters and low-quality reads were trimmed by Trimmomatic (v0.39).[21] All the remaining qualified reads were mapped to the *Rattus norvegicus* genome (Rnor_6.0) using HiSat2 (v2.1.0).[22] Then, FeatureCounts (v1.6.5)[23] was employed for gene quantification to build an expression matrix.

The differential expression of genes was determined by calculating fold changes using the normalized value of each group with the R package "DESeq2",[24] and the fold change values >1.3 and <0.77 at $p < 0.05$ were used as the threshold values. To further identify the pathways enriched in different groups, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to enrich the biological functions of differentially expressed genes (DEGs) with the R package "clusterProfiler".[25] In addition, the change in the immune microenvironment was examined using enrichment score-based algorithm xCell from RNA-seq data.[26]

16s rRNA gene sequencing and analysis

DNA was extracted from fecal content samples using the E.Z.N.A.® Bacterial DNA Kit (OMEGA, USA) to achieve an automatic and standardized DNA extraction across samples. Isolated DNA was kept at −20°C. Then, the variable 3–4 (V3–V4) regions of 16S rRNA was amplified using bacterial 16S rRNA gene-specific composite primers using the KAPA HiFi HotStart Ready Mix (KAPA Biosystems, Waltham, USA). Libraries were prepared using VANTS® DNA Clean Beads (Vazyme, Nanjing, China) and TruePrep® DNA Library Prep Kit V2 for Illumina (Vazyme, Nanjing, China). The following 16S amplicon PCR primers were used: F5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; R5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. Pooled amplicon libraries were sequenced using the HiSeq 150 bp

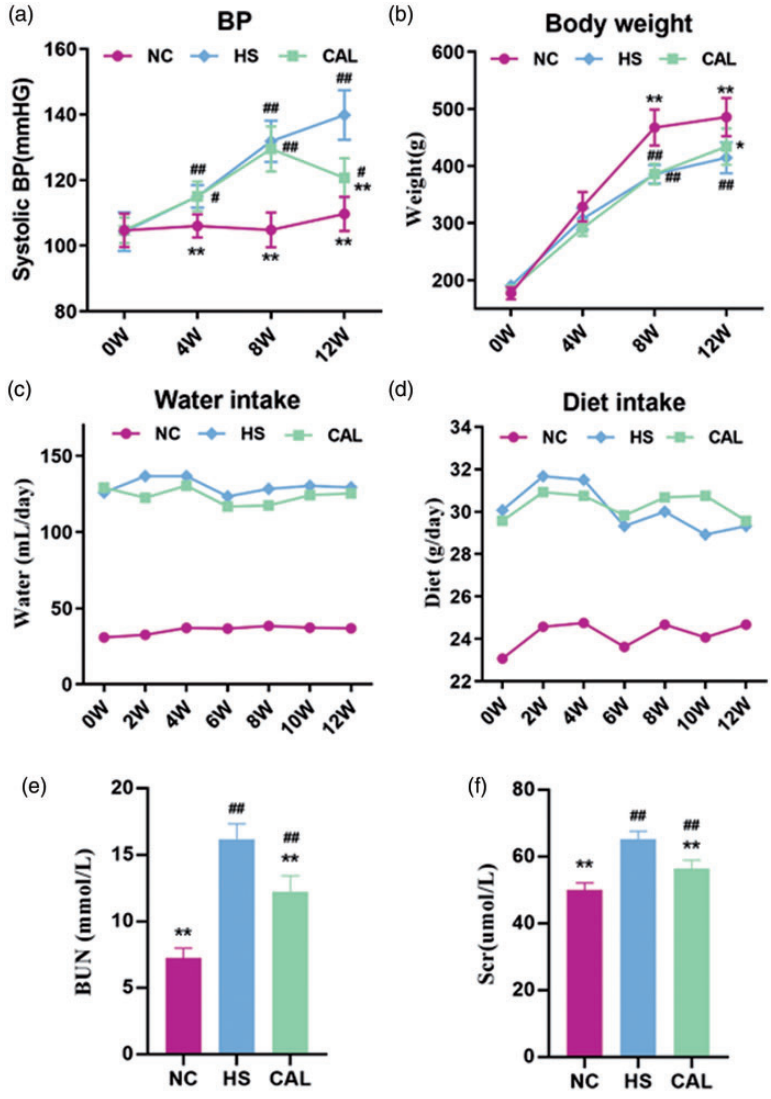

Figure 2. Sprague-Dawley (SD) Rats on a high-salt diet were predisposed to inflammation and hypertension. (a) Blood pressure (BP) was measured using the tail cuff method. (b) Body weight was measured throughout the experiment. The intake of water (c) and food (d) was recorded. Bar graph comparison of the levels of BUN (e) and Scr (f) in the three rat groups. *indicates groups compared with HS, and # indicates groups compared with NC. $^{*,\#}$represent $p < 0.05$; $^{**,\#\#}$represent $p < 0.01$; $^{***,\#\#\#}$ represent $p < 0.001$. The comparison results of CAL are marked on the right. (A color version of this figure is available in the online journal.)
BUN: blood urea nitrogen; Scr: serum creatinine.

paired-end sequencing (Illumina, USA) by Berry Genomics Co. Ltd. (Berry, Beijing, China). The sequencing data were further processed by QIIME (v1.9.1).[27] Briefly, sequences were clustered into phylotypes (Operational Taxonomic Units, OTUs) at 97% sequence identity using a uclust-based[28] closed-reference protocol, against the Greengenes database (August 2013 version).[29] Wilcoxon test was used to analyze the differences in microbiome composition between groups by relative microbiota abundance; $p < 0.05$ was considered to indicate statistical significance. After that, the PICRUSt software[30] was used to predict the function of the gut microbiota.

Analysis of host–microbiome cross-talk

To describe the host–microbiome crosstalk, Cytoscape (v3.8.2)[31] was used to construct gene co-expression networks of DEGs and OTUs present in the samples. Correlations between the microbiological composition of rat feces and BP data were also calculated. In brief, the Spearman's rank correlation coefficient was calculated for the quantitative data and the respective OTU abundance. Then, the Benjamini-Hochberg test was performed to determine the statistical significance of individual correlation coefficients; $p < 0.05$ was considered to indicate statistical significance.

Results

Blood pressure and pathology

The overall design details and physical data of the experiment are shown in Figure 1. In this study, we observed significantly higher BP levels in the HS group than in the NC group. This trend was already noted after four weeks (115.00 ± 3.41 mmHg vs. 106.00 ± 3.58 mmHg, HS vs. NC)

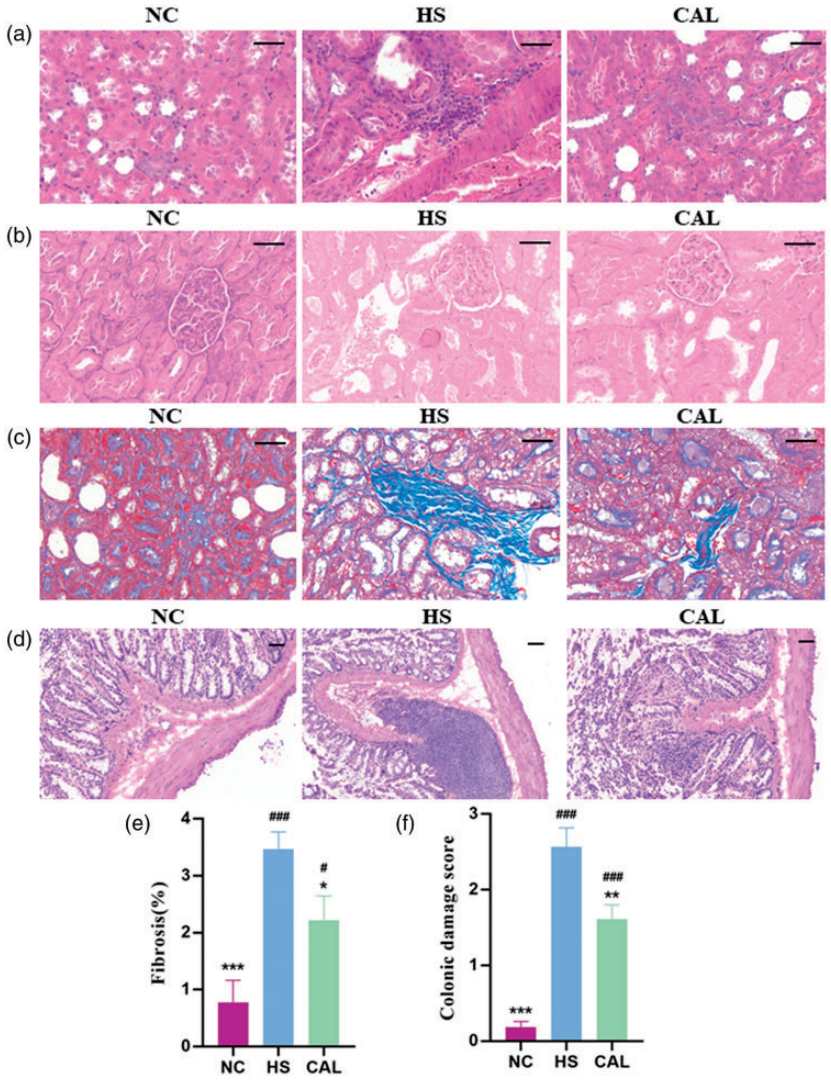

Figure 3. Pathology analyses of rats. Hematoxylin-eosin (HE)-stained kidney (a) and colon (d) tissue (scale bar = 40 μm) in each group. Periodic acid-Schiff (PAS) staining (b) and Masson's trichrome staining (c) of the kidney (scale bar = 40 μm) in each group. The renal fibrosis (e) and colonic damage scores (f) of each group. *indicates groups compared with HS and # indicates groups compared with NC. $^{*,\#}$ represent $p < 0.05$; $^{**,\#\#}$ represent $p < 0.01$; $^{***,\#\#\#}$ represent $p < 0.001$. (A color version of this figure is available in the online journal.)
NC: normal control; HS: high salt; CAL: calcitriol.

and was maintained throughout the study (139.83 ± 7.52 mmHg vs. 109.67 ± 5.20 mmHg, HS vs. NC). At the end of the experiment, the BP of the CAL group was lower than that of the HS group (120.67 ± 6.02 mmHg vs. 139.83 ± 7.52 mmHg, CAL vs. HS), but it was still significantly different than that of the NC group (120.67 ± 6.02 mmHg vs. 109.67 ± 5.20 mmHg, CAL vs. NC; Figure 2(a)). Rats in the HS group showed a higher food intake, water intake, BUN, and Scr levels but lower body weight than those in the NC group (Figure 2(b) to (f)). Compared with HS group, BUN and Scr levels in CAL group were significantly decreased (Figure 2(e) and (f)). Light microscopy of the stained sections showed histopathological changes in renal cortex among different groups (Figure 3(a) to (c)). Compared with NC group, kidneys in HS group revealed obvious, inflammatory cell infiltration, and increased fibrous tissues in the renal interstitium. CAL group significantly improved after four weeks of treatment. In the colon, HE-stained sections showed more inflammation in the HS and CAL groups than in the NC group; however, the degree of inflammation in the CAL group was lower than that in the HS group (Figure 3(d)). The renal fibrosis and colonic damage scores of the CAL group were significantly decreased compared with those of the HS group (Figure 3(e) and (f)).

mRNA analysis

The hierarchical clustering analysis (HCA) plot showed that samples from different groups were well isolated and biologically reproducible (Figure S1(a)). The t-distributed stochastic neighbor embedding (t-SNE) diagram shows a marked difference between the organs (Figure S1(b)). Among the groups, the NC and CAL groups were the most similar in kidney and colon (Figure S1(c) and (d)).

Based on the whole gene expression profile of the RNA-Seq, the comparison of gene expression between different

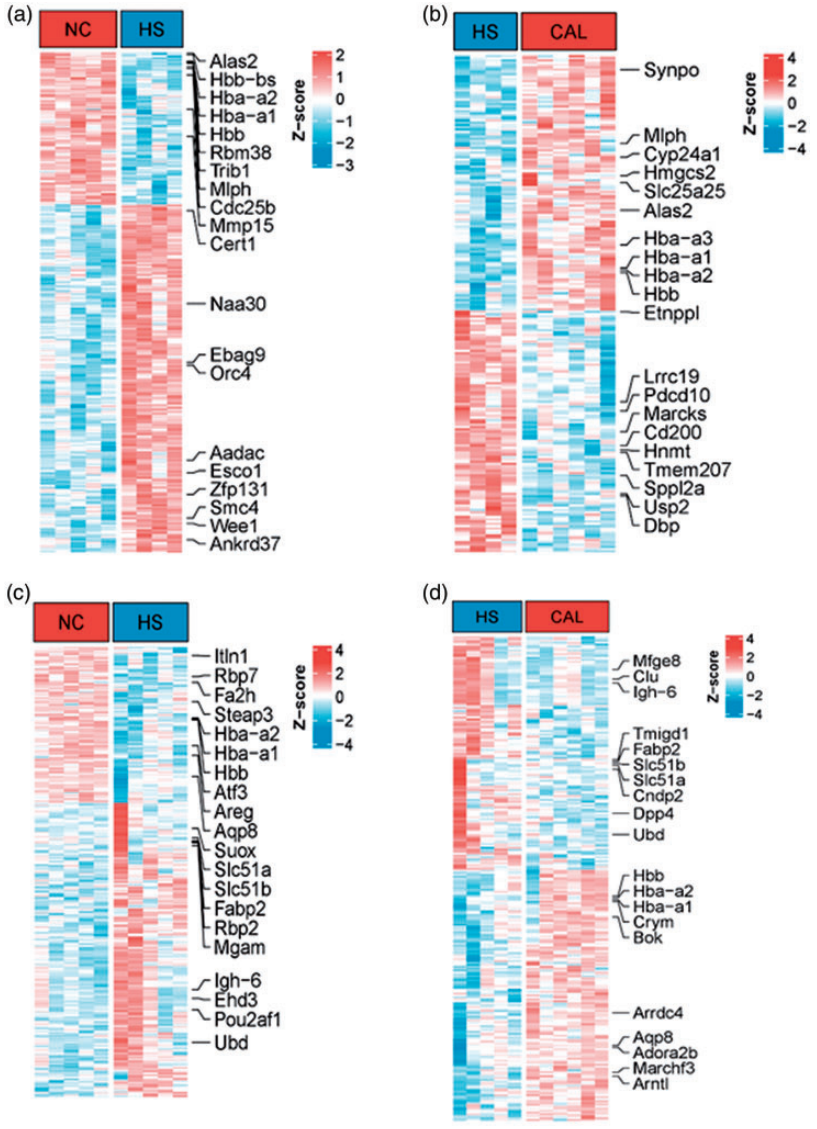

Figure 4. Transcriptome overview across high-salt diet-induced NC, HS, and CAL group development. (a–b) Top 20 differentially expressed genes (DEGs) among each comparison of the kidney. (c–d) Top 20 DEGs among each comparison of the colon. (A color version of this figure is available in the online journal.)
NC: normal control; HS: high salt; CAL: calcitriol.

groups was observed (Figure S1(e) and (f)). In the kidney, the comparison between the HS and NC groups showed 373 upregulated genes and 186 downregulated genes. The comparison between the CAL group and the HS group showed 249 upregulated genes and 274 downregulated genes (Supplementary Table S1). The top 10 significantly upregulated and downregulated genes are highlighted in Figure 4(a) and (b). In the colon, A total of 582 and 409 DEGs were identified in the comparisons of HS vs. NC and CAL vs. HS (Supplementary Table S2), respectively. The top 10 significantly upregulated and downregulated genes are highlighted in Figure 4(c) and (d).

Gene expression profiling and functional analysis of the kidney

Of the DEGs between the HS and NC groups and CAL and HS groups, 20 and 23, respectively, were identified as transcription factors (TFs). *Tsc22d2*, *Nfe2l2*, and *Mbd2* were highly expressed in the HS group, whereas *Hnf4a* and *Cyp24a1* were highly expressed in the CAL group. Moreover, gene expression profiling revealed increased expression of immune-related genes in the HS group, including *Tlr3*, *Malt1*, and *Mapk1*. Notably, decreased expression of *Cd48* and *Mme* was observed in the CAL group. The Gene Ontology Biological Process (GOBP) analysis identified several functions of the DEGs, and the predominant functions of the DEGs between the HS and NC groups included regulation of cellular amide metabolic process, translational initiation, and regulation of cellular amide metabolic process (Figure 5(a)). Additionally, genes related to phospholipid biosynthetic process, positive regulation of cellular protein localization were predominantly enriched between the HS and CAL groups (Figure 5(c)). The KEGG database is a collection of pathway maps representing molecular interaction, reaction, and relation networks. The KEGG pathway analysis identified DEGs

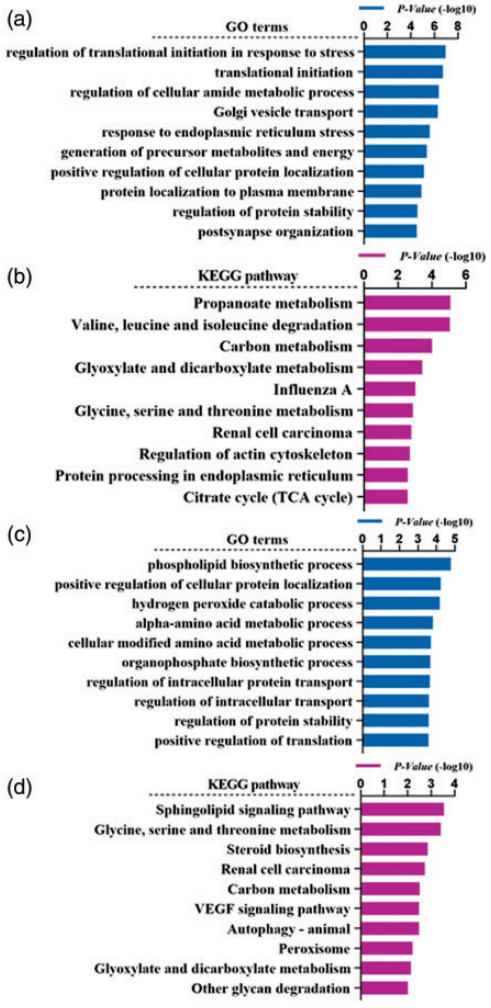

Figure 5. Kidney transcriptome analysis. (a–b) Gene ontology (GO) biological process analysis and KEGG pathway analysis is applied between the NC and HS group. (c–d) GO biological process analysis and KEGG pathway analysis is applied between the HS and CAL group. (A color version of this figure is available in the online journal.)
GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

between the NC and HS groups mainly related to propanoate metabolism and regulation of actin cytoskeleton (Figure 5(b)). Moreover, the DEGs between the HS and CAL groups were identified to be significantly enriched in several metabolic pathways, such as glycine, serine, and threonine metabolism and glyoxylate and dicarboxylate metabolism (Figure 5(d)).

Gene expression profiling and functional analysis in the Colon

Of the DEGs between the HS and NC groups and CAL and HS groups, 33 and 19, respectively, were identified as TFs. *Acer3*, *Cldn3*, and *Cldn23* were downregulated in the HS group. *Msn* was highly expressed in the HS group, whereas *Tjp2*, *Cldn4*, and *Cldn23* were upregulated in the CAL group. *Msn* was downregulated in CAL than in HS. Immune-related genes such as *Vcam1*, *Casp2*, *C1r*, *Cd44*, *Psmb9*, and *CD74* were highly expressed in HS than in NC. *C1r*, *Psmb9*, *Cd74*, *Cd81*, and *Cd83* were downregulated

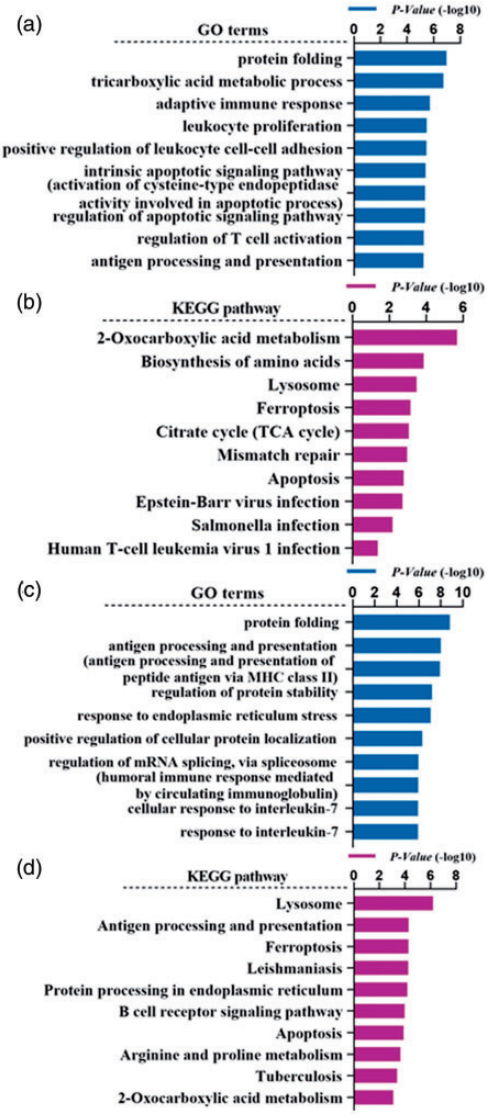

Figure 6. Colon transcriptome analysis. (a–b) GO biological process analysis and KEGG pathway analysis is applied between the NC and HS group. (c–d) GO biological process analysis and KEGG pathway analysis is applied between the HS and CAL group. (A color version of this figure is available in the online journal.)
GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

in the CAL group. The GOBP analysis identified several functions of DEGs between HS and NC, including adaptive immune response, regulation of apoptotic signaling pathway, and regulation of T cell activation (Figure 6(a)). The KEGG analysis also identified DEGs significantly enriched in several pathways related to immune system function, including immune response to Epstein-Barr virus infection, Salmonella infection, and human T-cell leukemia virus 1 infection (Figure 6(b)). The other significantly enriched pathways associated with the DEGs between HS and CAL included 2-oxocarboxylic acid metabolism, apoptosis, and B-cell receptor signaling pathway (Figure 6(d)).

Changes in the immune microenvironment of the kidney and colon

As many differentially expressed genes and pathways were immune related, we next sought to examine the changes in

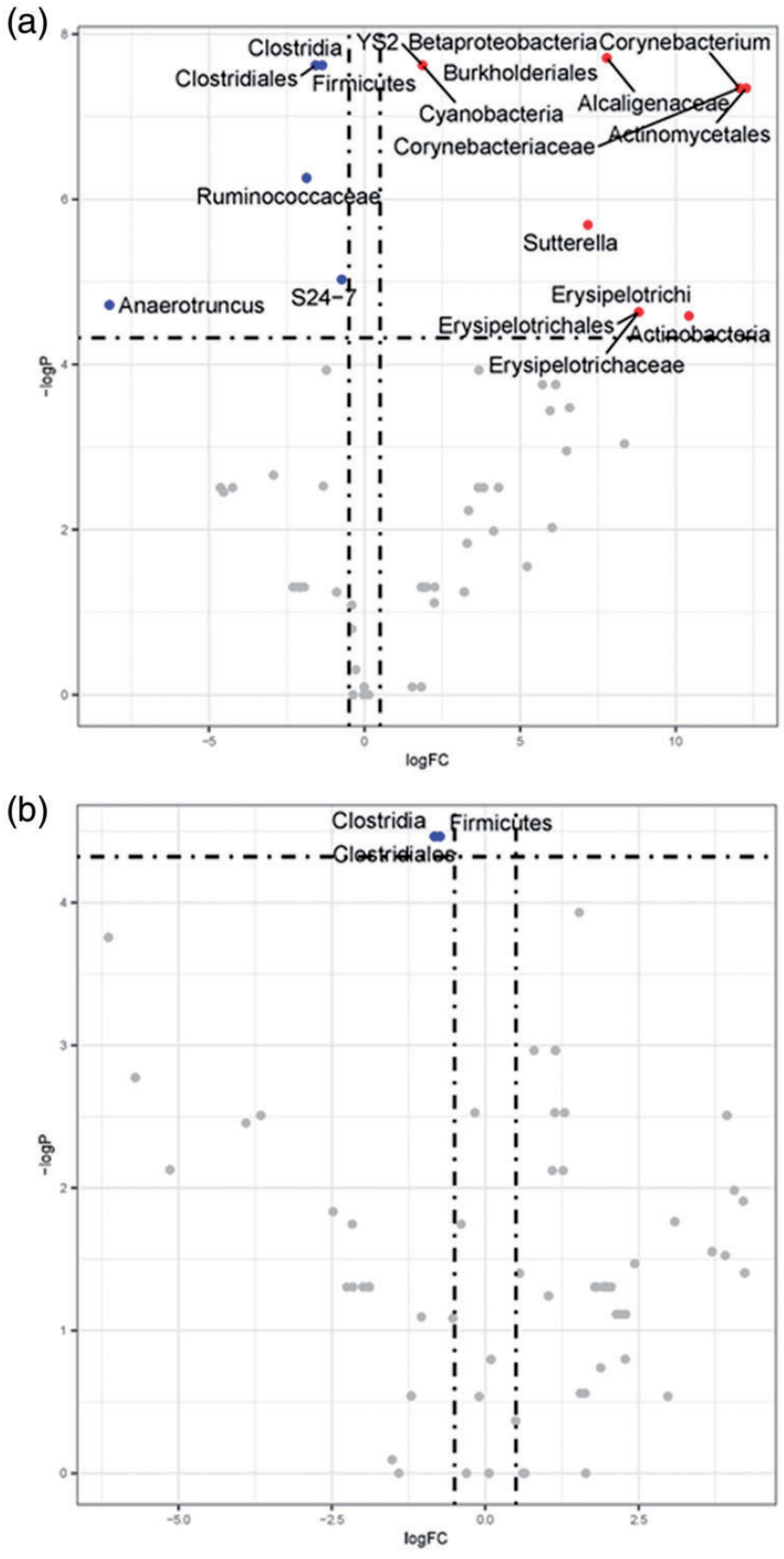

Figure 7. Effect of a high-salt diet on the microbiota of rat. (a) Volcano plot analysis of microbiota changes between the NC and HS group. (b) Volcano plot analysis of microbiota changes between the HS and CAL group. (A color version of this figure is available in the online journal.)

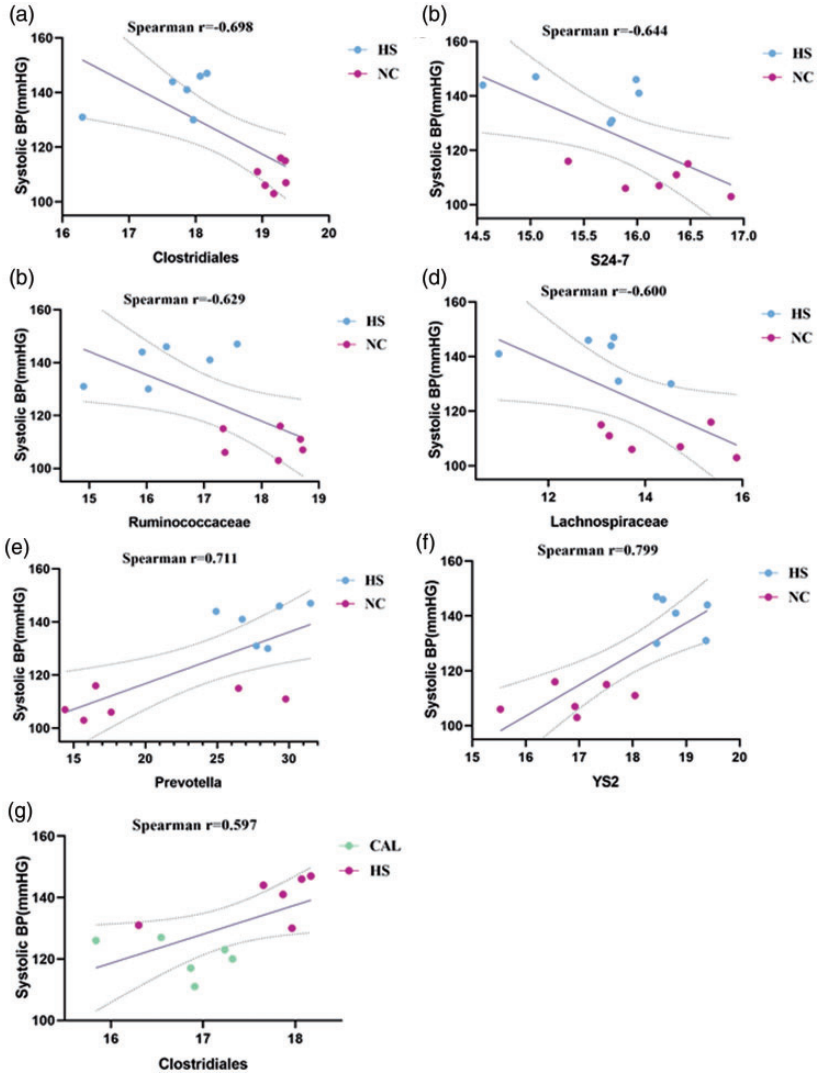

Figure 8. Correlation between blood pressure (BP) and bacterial taxa. (a–g) Spearman's rank correlation coefficient was calculated for the BP values for 12 weeks and the respective OTU abundance. (A color version of this figure is available in the online journal.)

the immune microenvironment across NC, HS, and CAL by RNA-seq deconvolution. The heatmap of the relative expression of genes associated with immune cells is shown in Figure S2. Remarkably, $CD8^+$ and $CD4^+$ T cells were hyperactivated in the colon of the HS group rats, whereas natural killer (NK) cells were more abundant in the NC and CAL groups.

Microbiome profile

To determine the effect of salt and calcitriol on the microbiome composition, we analyzed fecal pellets from CAL, HS, and NC groups by 16S rRNA amplicon sequencing. A total of 16.64 million 16S rRNA amplicons were obtained from 18 bacterial samples attached to rat colonic mucosa, and an average of 924,600 reads were sequenced per sample. Figure S3(a) indicates the eight most abundant phyla. The principal coordinate analysis (PCOA) plot using the Bray-Curtis index showed differences in the composition of the microflora among the groups (Figure S3(b) and (c)). The Shannon–Wiener diversity index and Chao1 index indicated no significant difference in bacterial richness and diversity among the three groups (Figure S3 (d)). The PICRUSt tool was used to predict the functional profiles of the gut microbiota. The gut microbiota pathway functions showed that several pathways in the gut microbiome changed significantly between the HS and CAL groups, especially the pathways associated with arginine and proline metabolism, fatty acid metabolism, phenylalanine metabolism, and apoptosis (Figure S3(e)).

The volcanic plots obtained by Wilcoxon test analysis show the results of differences among different levels of microbiome (Figure 7(a) and (b)). At the phylum level, the HS group showed more abundant of *Cyanobacteria* and less abundant of *Firmicutes* of NC versus HS. Moreover, the high-salt diet promoted higher abundant of *Alcaligenaceae* and less abundant of *Ruminococcaceae* (NC versus HS). In contrast, the abundance of *Firmicutes* and *Clostridia* was reduced in the CAL group (HS versus CAL). At the genus level, *Sutterella* and *Corynebacterium* showed higher abundance in the HS group (NC vs. HS). However, no difference was found at the genus level in the comparison between the HS and CAL.

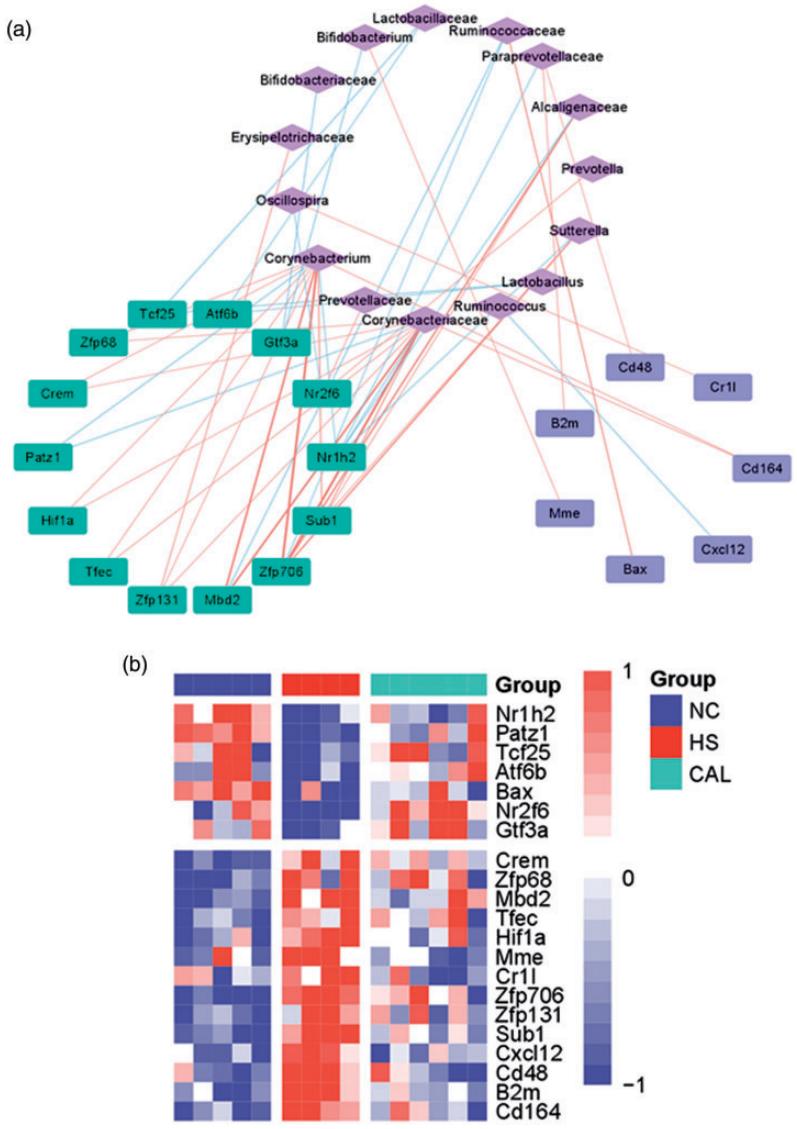

Figure 9. Co-abundance analysis of microbiome and kidney transcriptome. (a) The core panel of transcription factors (TFs) and immune-related genes determine the main link between the gut microbiota and the differentially expressed genes (DEGs) in the kidney. (b) Heatmap of DEGs involved in the crosstalk. (A color version of this figure is available in the online journal.)
NC: normal control; HS: high salt; CAL: calcitriol.

Notably, a linear correlation analysis between the microflora and BP identified seven unique taxa at the order, family, and genus level (Figure 8(a) to (g)). In the comparison between the HS and NC groups, the abundance of *Lachnospiraceae*, *Ruminococcaceae*, *S24-7*, and Clostridiales was negatively correlated with BP, whereas that of *Prevotella* and *YS2* was positively correlated with BP. In the comparison between the CAL and HS groups, the abundance of Clostridiales was identified to be positively correlated with BP.

Crosstalk between the gut microbiota and the host

The crosstalk between the gut microbiota and host has attracted considerable attention owing to its involvement in diverse diseases. To examine the host–microbial crosstalk caused by intestinal flora dysregulation, we identified co-expressed immune genes and TFs associated with abundant bacterial OTUs. Crosstalk was observed between 23 gut microbes and 173 immune genes in the kidney and between 20 gut microbes and 113 immune genes in the colon. On the basis of Pearson correlation, the immune-related genes and TFs of DEGs were used to construct co-expressing networks (Figure 9(a) and 11(a)). We also used these DEGs to construct a heatmap (Figure 9(b) and 11(b)). Remarkably, crosstalk was observed between the microbiome and multiple clock-related TFs that regulate circadian rhythm, such as *Arntl*, *Atf4*, and *Npas2*. The circadian clock regulates the rhythmic oscillations of physiological processes during the course of the day. These findings suggest that the intestinal flora plays an indispensable role in the host's circadian rhythm. The protective mechanism of calcitriol may partly affect the excretion of sodium by restoring the regulation of these circadian signals, thereby further affecting the BP.

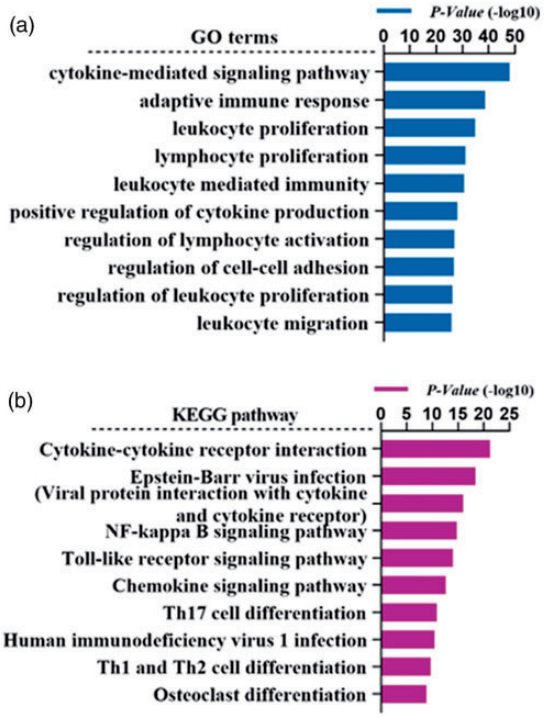

Figure 10. Co-abundance analysis of the kidney transcriptome. (a–b) The gene ontology (GO) biological processes and KEGG pathways of co-abundance immune-related genes in the kidney. (A color version of this figure is available in the online journal.)
GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

To determine the crosstalk between gene patterns and the gut microbiome, we performed a functional analysis of these co-expressed immune genes. The pathways associated with the genes expressed in the kidney are shown in Figure 10(a) and (b). The crosstalk-related genes expressed in the colon were associated with three predominant biological processes—cytokine-mediated signaling pathway, leukocyte proliferation, and lymphocyte proliferation—and a predominant signaling pathway—Epstein Barr virus infection (Figure 12(a) and (b)). Remarkably, the pathways associated with the intestinal mucosa and inflammation—Th1 and Th2 cell differentiation and Th17 cell differentiation—were enriched in both tissues. In addition, the NF-Kappa B signaling pathway, known to negatively regulate immune activation in response to bacterial stimulation associated with vitamin D, was also enriched.[32] This suggests that crosstalk between host transcriptome and microbiome may be associated with preventing bacterial invasion and host infection by mediating barrier function and intestinal homeostasis.

Discussion

Previous studies have established that a high-salt diet induces hypertension in rats and causes kidney and intestinal damage.[33–36] In our study, we created a high-salt diet rat model using 8% NaCl in the diet and observed renal and colonic inflammation and hypertension. Environmental factors such as diet and microbiome composition play a crucial role in the development of inflammatory diseases.[37] Here, we showed that a high-salt diet regulates the composition and function of the intestinal flora. A series of changes in immune and inflammatory genes were found throughout the transcriptome. The immune dysfunction promotes the proinflammatory state of the colon and kidney and is associated with the changes in the intestinal flora. Decades of research have shown that vitamin D plays a key role in regulating both adaptive and innate immunity.[38] In our study, we observed the effects of salt and calcitriol on the composition of the intestinal flora and the immune system and explored the microbial patterns associated with this immune dysfunction. We found that immune dysfunction is mainly related to the correlation between the intestinal microbiome, intestinal inflammation, and the circadian clock, and the intestinal microbiome is a key mediator in this process.

Recent evidence shows that gut microbiota plays a role in the development of cardiovascular diseases, including hypertension.[39] Germ-free mice are resistant to hypertension and vascular dysfunction and have less renal and vessel infiltration of immune cells after angiotensin II infusion.[40] Salt-sensitive hypertension is associated with gut dysfunction and elevated intestinal permeability.[6] In our study, 16S rRNA gene sequencing results suggested altered gut microbiota composition in the HS group compared to the NC and CAL groups. The PCOA plot revealed an overall separation among groups. Our results showed that consumption of high-salt diet promoted higher abundance of phylotypes belonging to *Alcaligenaceae* and *Sutterella* and a decrease abundance of *Ruminococcaceae*. Importantly, the abundance of the *Alcaligenaceae* family has previously been linked to immunomodulation.[41] *Ruminococcaceae* has been clearly shown to negatively correlate with intestinal inflammation.[42] Moreover, the abundance of *Sutterella* have been frequently associated with pro-inflammatory cytokines.[43] However, we only found a few taxa's relative abundance was appreciably different between the HS and CAL group. Fox example, Clostridia, a dominant class of commensal microbe, can induce colonic regulatory T (Treg) cells.[44] We speculate that its decrease in the CAL group may be due to its antagonistic effect on the regulation of Treg cells by calcitriol. These findings may indicate that major changes in a few specific taxa determine the differences between cases. We predicted metagenomic KEGG pathways using PICRUSt; multiple metabolic pathways were affected by a high-salt diet, consistent with previous findings.[37] One study showed that gut microbiota dysbiosis leads to abnormal accumulation of amino acids, which are released into the peripheral blood and activate the innate immunity and lead to apoptosis.[45] As reported before, in our study, phenylalanine metabolism and apoptosis pathway were significantly different between the HS and CAL groups. In addition, recent research indicates that a high-salt diet disrupts the development and function of NK cells, resulting in a decreased proportion and absolute number of NK cells.[46] This finding is in accordance with our previous findings on immune microenvironment—the number of NK cells was lower in the HS group than in the NC group. Remarkably, a comparison of the CAL and HS groups showed that NK cells were accumulated in the

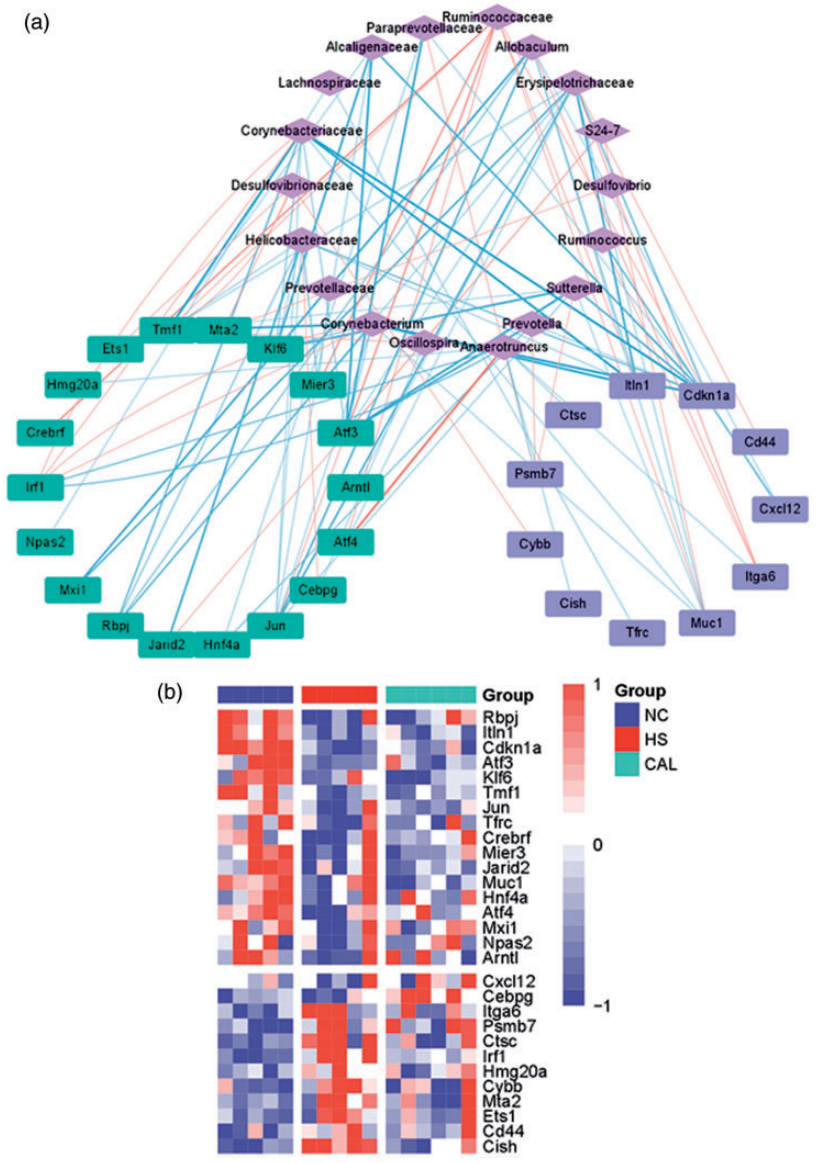

Figure 11. Co-abundance analysis of microbiome and colon transcriptome. (a) The core panel of transcription factors (TFs) and immune-related genes determine the main link between the gut microbiota and the differentially expressed genes (DEGs) in the colon. (b) Heatmap of DEGs involved in the crosstalk. (A color version of this figure is available in the online journal.)

CAL group, indicating that calcitriol restores the dysregulated NK cells. These findings highlight the relationship between the gut microbiota and disease activity and the protective effect of calcitriol.

We identified seven microbial taxa at the level of order, family, and genus that were significantly associated with BP. To our knowledge, there are only a few very recent studies that have reported a high-salt diet-mediated association between the intestinal microbiome and cardiovascular risk. Some previous studies found that *Prevotella* colonization caused weight loss and aggravated intestinal epithelial inflammation in a mouse model of colitis.[47] Li *et al.*[48] found that *Prevotella* was a dominant taxon in the hypertension cohort and suggested that it is a causal factor of inflammation and hypertension. This finding is consistent with the present results. Notably, we found that the abundance of certain SCFA-producing bacteria (*Lachnospiraceae* and *Ruminococcaceae*), both belonging to clostridia cluster XIVa, has a negative correlation with BP, which is consistent with previous studies.[49–51] Clostridia cluster XIVa is one of the three main groups of strict anaerobes in the intestine that accounts for the majority of the butyrate production, which has been shown to have a protective effect against colitis.[52] One study quantified SCFAs in mice on a high-salt diet and found that only butyric acid levels were significantly changed.[37] In addition, it has been found that butyrate causes intestinal macrophages to downregulate the production of lipopolysaccharide-induced proinflammatory cytokines (i.e. NO, IL-6, and IL-12),[53] further supporting the role of butyrate as an anti-inflammatory metabolite. However, Clostridiales was the only microbial taxon with a significant correlation with BP in the CAL and HS groups. Very few studies have reported the direct effects of vitamin D on bacteria. One

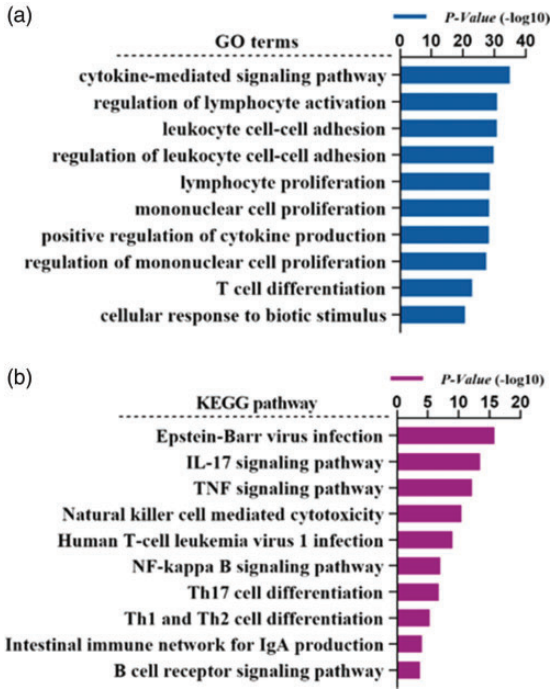

Figure 12. Co-abundance analysis of the colon transcriptome. (a–b) The gene ontology (GO) biological processes and KEGG pathways of co-abundance immune-related genes in the colon. (A color version of this figure is available in the online journal.)
NC: normal control; HS: high salt; CAL: calcitriol.

study has shown that vitamin D inhibits the growth of certain mycobacteria *in vitro*, but this has not been verified.[54] Overall, the findings suggest that high-salt diets change the composition and function of the gut microbiota and reduce butyric acid, which affects BP and intestinal inflammation. The protective effect of vitamin D may be mediated indirectly by immunological properties, with little direct effect on the intestinal flora.

The homeostasis of vitamin D metabolism mainly occurs in the intestines and kidneys.[55] Although many of the genes that are expressed and associated with vitamin D action in kidney are well defined, the biological processes and genes that mediate the process of intestinal calcium absorption are not fully understood.[56] As some studies have found, *Cyp24a1* is upregulated by 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$),[57] and this may only occur specifically in the renal tissue,[58] which is consistent with our study. Intestinal calcium absorption is mainly through transcellular calcium absorption and the paracellular pathway. In some gene knockout experiments, $1,25(OH)_2D_3$ was found to restore the expression of active calcium transport-related genes (*Trpv6*, *Calb9K*, and *Atp2b1*) in the case of Ca^2+ deficiency.[56,59] In our study, there was no change in the expression of active calcium transport-related genes. As previously reported, this may be because the high-salt diet causes intestinal inflammation and fibrosis,[60–62] which may disrupt normal intestinal function and then cause $1,25(OH)_2D_3$ is failed to regulate the genes related to calcium transport. In more recent studies, paracellular Ca^{2+} absorption was shown to be associated with the claudin protein family.[63] Studies by Zhang *et al.* found that *Cldn2* is the target of vitamin D receptor.[64] One study found that $1,25(OH)_2D_3$ up-regulated the expression of *Cldn2* and *Cldn12* in Caco-2 cells.[65] It is worth noting that an analysis of claudin expression in murine intestine showed that the absence of some claudins may be compensated by the maturation of other claudins.[66] In our study, multiple claudin protein family genes were altered, such as *Cldn3*, *Cldn4*, and *Cldn23*. Thus, these findings suggest that calcitriol regulates the claudin protein family genes associated with paracellular calcium transport in high-salt diet-induced hypertension. However, the specific mechanism needs further experimental verification.

The intestinal barrier acts as a gateway to enable a bidirectional passage of numerous metabolites and immune signals between the gut and circulation via the transcellular and paracellular transport mechanisms.[67] Studies have shown that changes in the composition of the intestinal flora caused by a high-salt diet damage the intestinal barrier function and trigger the immune response and the production of proinflammatory cytokines.[68] Studies have confirmed that a high-salt diet polarizes immune cells toward an inflammatory phenotype and enhances hypertension.[69] High BP causes T cells to infiltrate the kidney and perivascular space and release inflammatory cytokines that promote renal and vascular dysfunction.[70,71] We observed that a high-salt diet resulted in the downregulation of *Acer3* in the colon; Acer3 deficiency aggravates intestinal epithelial damage, weight loss, and systemic inflammation,[72,73] which is consistent with our results. As previously reported, in our study, several DEGs associated with immune response and inflammation in the kidney and colon, respectively, were identified in the comparison of gene expression between the HS and NC groups.

Multiple genes were upregulated in the HS group, such as *Vcam1*,[74] *Cd44*,[75] and *Cd74*[76] in the colon and *Malt1*,[77] *Tlr3*,[78] and *Mapk1*[79] in the kidney, all of which play a key role in inflammation. *Msn* is a member of the Ezrin-radixin-moesin (ERM) family, which are cross-linkers between transmembrane receptors and cortical actin filaments.[80] Some studies have confirmed that the activation of ERM protein is a signal of increased endothelial permeability and an inflammatory-like response.[81–83] In our study, calcitriol reversed the up-regulated expression of *Msn* induced by a high-salt diet, indicating the improved barrier function. Similarly, a recent study confirmed that increased mRNA levels of *Tjp2* indicates improved gut barrier function,[84] which is consistent with our results. In the KEGG pathway analysis, multiple metabolic pathways such as glutathione metabolism, sulfur metabolism, and fatty acid metabolism were enriched. Alterations in cellular metabolism play an important role in controlling and steering the inflammatory state of both endothelial cells and immune cells, and this change has been shown to be related to hypertension in multiple studies.[85–87] Many studies have demonstrated the brain–gut connection pathway of hypertension, but relatively little is known about the role of gut–kidney connection, in particular, whether changes in renal function are associated with increased intestinal permeability and changes in the expression of inflammatory genes. Our results present candidate pathways and genes to explore the relationship between metabolism, inflammation, and hypertension on the gut–kidney axis.

Furthermore, we explored the relationship between gut microbiome, immune genes, and TFs. The results of OTU-TF co-expression analysis showed that circadian rhythm signal genes such as *Arntl*,[88] *Atf4*,[89] and *Npas2*[90] were associated with intestinal flora. Diet has been proven to affect the circadian rhythm, and previous studies have shown a relationship between these signals and BP.[91] A previous study found that clock genes (*Clock* and *Per1*) affect sodium reabsorption, and the destruction of these clock proteins leads to the deterioration of hypertension pathology.[92,93] The results of the co-expression analysis of OTUs and immune genes showed that some gut microbes were closely associated with the expression of immune-related genes. According to the pathway enrichment analysis, the co-expressing OTUs and immune genes were mainly associated with Th17 cell differentiation and Th1 and Th2 cell differentiation. Intestinal $CD4^+$ T helper (Th) cells are key mediators of mucosal immunity and are classified as Th1, Th2, and Th17 cells according to their effector functions.[94] A high sodium intake promotes the activation of Th-17 cells, leads to a proinflammatory immune response, and contributes to the development of hypertension.[95] An animal study showed that under a high-sodium diet, germ-free mice showed more anti-inflammatory T regulatory cells, and the number of cells was inversely proportional to that of Th17 cells.[95] This indicates that sodium-induced activation of Th17 cells and immune activation may be mediated by the intestinal flora. Studies have discovered the role of vitamin D in immune regulation.[10–12] In the CAL group, we observed a reduction in colon and kidney inflammation and altered expression of several immune-related and inflammation-related genes, such as *Hnf4a*,[96] *Cd48*,[97] and *Mme*.[98] This suggests that calcitriol can inhibit inflammation and immune activation in high-salt diet-induced hypertension, and this improvement may be related to the interaction of gut microbiome. Indeed, accumulative evidence suggests a role of intestinal microbiota in gene expression and pathogenesis. Cane *et al*. have reported that pro-inflammatory *Escherichia coli* could regulate the expression of *VEGF* and cause inflammatory bowel disease through the induction of the adhesin-dependent activation of decay accelerating factor signaling.[99] Weger *et al*. found that the altered microbiota-derived metabolites affect the expression of multiple tissue genes in the host, including some rhythm signal genes,[100] which is consistent with our results. The mechanisms by which the intestinal flora and their metabolites regulate BP are complex. Some studies have found that changes in the intestinal flora of patients with kidney disease affect the production of intestinal toxins, which enter the circulatory system through the intestinal barrier and cause systemic effects.[101] In addition to inflammation, there are more unexplained interactions between the intestinal flora and the diurnal signals in regulating BP, and the protective effects of calcitriol require further confirmation.

Taken together, the present findings suggest that diet and calcitriol play a key role in shaping the gut microbial communities and transcriptome expression, which influence the host physiology and predisposition to disease.

Conclusions

The strengths of our study are the synchronous analyses of transcriptome and microbiome and identification of the target damage locations of hypertension; we also attempted to explain the reciprocal effects of vitamin D, gut barrier function, microbiome, and immune responses. Several changes in immune and inflammatory genes were found throughout the transcriptome. 16S rRNA sequencing revealed changes in the abundance and function of the gut microbiota. We observed the effects of high dietary salt levels and calcitriol on the composition of the intestinal flora and the immune system and explored the microbial patterns associated with this immune dysfunction. The results of the co-expression analysis of OTUs and intestinal DEGs indicate that some intestinal microbes are closely associated with the expression of immune-related genes and clock genes in the colon and kidney, and the intestinal microbiome is a key mediator in this process.

AUTHORS' CONTRIBUTIONS

RD, YY, and YJ established the model and completed the experiments; RD, ZX, and SZ analyzed the data and performed the bioinformatics analysis; YJ, XZ, KX, and XB reviewed the conclusions; RD, ZX, XZ, and SZ presented the idea of this paper, drafted and revised the article.

DECLARATION OF CONFLICTING INTERESTS

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DATA AVAILABILITY

The datasets used and/or analyzed during the current study have been uploaded to the GEO database (GSE184844) and SRA database (PRJNA766530).

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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