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The Colonic Microbiome and Epithelial Transcriptome Are Altered in Rats Fed a High-Protein Diet Compared with a Normal-Protein Diet[1–3]

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Abstract

Background: A high-protein diet (HPD) can produce hazardous compounds and reduce butyrate-producing bacteria in feces, which may be detrimental to gut health. However, information on whether HPD affects intestinal function is limited.

Objective: The aim of this study was to determine the impact of an HPD on the microbiota, microbial metabolites, and epithelial transcriptome in the colons of rats.

Methods: Adult male Wistar rats were fed either a normal-protein diet (20% protein, 56% carbohydrate) or an HPD (45% protein, 30% carbohydrate) for 6 wk (n = 10 rats per group, individually fed). After 6 wk, the colonic microbiome, microbial metabolites, and epithelial transcriptome were determined.

Results: Compared with the normal-protein diet, the HPD adversely altered the colonic microbiota by increasing ($P < 0.05$) *Escherichia*/*Shigella*, *Enterococcus*, *Streptococcus*, and sulfate-reducing bacteria by 54.9-fold, 31.3-fold, 5.36-fold, and 2.59-fold, respectively. However, the HPD reduced *Ruminococcus* (8.04-fold), *Akkermansia* (not detected in HPD group), and *Faecalibacterium prausnitzii* (3.5-fold) ($P < 0.05$), which are generally regarded as beneficial bacteria in the colon. Concomitant increases in cadaverine (4.88-fold), spermine (31.2-fold), and sulfide (4.8-fold) ($P < 0.05$) and a decrease in butyrate (2.16-fold) ($P < 0.05$) in the HPD rats indicated an evident shift toward the production of unhealthy microbial metabolites. In the colon epithelium of the HPD rats, transcriptome analysis identified an upregulation of genes ($P < 0.05$) involved in disease pathogenesis; these genes are involved in chemotaxis, the tumor necrosis factor signal process, and apoptosis. The HPD was also associated with a downregulation of many genes ($P < 0.05$) involved in immunoprotection, such as genes involved in innate immunity, *O*-linked glycosylation of mucin, and oxidative phosphorylation, suggesting there may be an increased disease risk in these rats. The abundance of *Escherichia/Shigella*, *Enterococcus*, and *Streptococcus* was positively correlated (Spearman's $\rho > 0.7$, $P < 0.05$) with genes and metabolites generally regarded as being involved in disease pathogenesis, suggesting these bacteria may mediate the detrimental effects of HPDs on colonic health.

Conclusion: Our findings suggest that the HPD altered the colonic microbial community, shifted the metabolic profile, and affected the host response in the colons of rats toward an increased risk of colonic disease. *J Nutr* 2016;146:474–83.

Keywords: microbial community, bacterial metabolites, epithelial response, colonic disease risk, colonic microbiome, epithelial transcriptome, high-protein diet

Introduction

High-protein diets (HPDs)[6] have been related to an increased risk of colon disease (1). Both colonic microbiota and gene expression are involved in regulating colonic health (2, 3). However, their response to HPDs remains unclear. Clinical trials have shown that an HPD affects the fecal bacteria composition and increases the concentration of the harmful N-nitroso compounds (NOCs), while decreasing the concentration of the beneficial compound butyrate in human feces (4). The fecal

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[3] Supplemental Tables 1–5 and Supplemental Figures 1–2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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abundance of *Bifidobacterium* and *Roseburia/Eubacterium rectale*, which are generally regarded as beneficial (5), was reduced in adults consuming HPDs (4, 5). A recent study showed that an HPD reduced the quantity of *Clostridium coccoides*, *Clostridium leptum* groups, and *Faecalibacterium prausnitzii* in the colonic lumen of rats (6). The decreases in bacteria within *Bifidobacterium*, *Roseburia/E. rectale*, and *C. coccoides* were related to the reduction of butyrate production (5). Decreases in *E. rectale* and *F. prausnitzii* have been observed in patients with colorectal cancer (7), suggesting the relevance of these bacteria in colonic health. NOCs could induce DNA damage and are potential carcinogens (8), whereas butyrate exerts anti-inflammatory properties, regulates energy metabolism in the colon, and may downregulate carcinogenesis by promoting apoptosis in colorectal cancer cells (3). These results, although mostly from fecal analysis, provide evidence for the potentially unfavorable effects of HPDs on the gut health (9, 10). Furthermore, gut microbiota exerts an influence on colonic health via both the community composition and microbial metabolism products (11). Thus, it is important to understand the impact of HPDs on the colonic microbiome in both composition and metabolic profile when evaluating the potential risk of HPDs on the colonic health.

It has been also reported that HPDs could affect intestinal gene expression. Recent research has shown that an HPD affected some colonic bacteria and downregulated IL-6 gene expression but did not affect the gene expressions of Toll-like receptors or inflammatory cytokines by gene-targeted qPCR analysis in the colon of rats (12). This suggests that HPDs may have the potential to adversely affect immune homeostasis. Given that many factors could contribute to gut immune homeostasis, in addition to an immune component, factors that are involved in other processes such as oxidative stress or carcinogenesis are also important in shaping gut immune homeostasis. Thus, it is crucial to reveal the changes of all possible factors involved in the colon health and understand the overall picture of the epithelial responses to an HPD. Furthermore, it is necessary to understand the relation between gut microbiota and epithelial responses because it may implicate potential mechanisms for regulating colon health by gut microbiota.

In the present study, we integrated the microbiome and transcriptome analyses to test the hypothesis that an HPD may lead to colonic disease risk. The colonic microbiome and its metabolic profile under the HPD were investigated to uncover the structural changes of the microbial community. The epithelial transcriptome was evaluated to dissect the overall colonic response, and the correlations between host gene expressions and microbiota were also revealed, aiming to understand the impact of HPDs on colonic health.

Methods

Animal, diet, and experimental design. The experiments were conducted in compliance with the Chinese regulations on the protection of experimental animals, following the protocol approved by the Ethical Committee of Nanjing Agricultural University, Nanjing, China. Twenty adult male Wistar rats (Qinglongshan Animal Center, Nanjing, China) weighing 180–200 g were maintained individually in polycarbonate cages with free access to water and under room temperature (mean ± SE: 22 ± 4°C) with a mean ± SE relative humidity of 50% ± 15% in a 12-h:12-h light/dark cycle. Rats were fed with the standard normal-protein diet (NPD) during the 1-wk acclimation period. Then they were randomly allocated to 2 groups with 10 rats each and offered 1 of 2 isoenergetic diets: an NPD (20% protein, 56% carbohydrate) or an HPD (45% protein, 30% carbohydrate) (**Supplemental Table 1**). The sample size chosen was based on a previously published report that demonstrated, using HPD diet trials, the effect of an HPD on intestinal environment in rats (13). Food residues were recorded daily and fresh food was provided at the end of the daily light period. No signs of illness were observed for all animals during the 6-wk experiment, and all animals were included for further analysis. At the end of experiment (42 d), rats were feed-deprived overnight, weighed, and anesthetized with diethyl ether. Luminal contents from the proximal colon were collected by expulsion. Colonic contents were stored at −80°C before further DNA extraction or metabolite measurements, and colon tissues were snap frozen immediately and stored at −80°C for microarray analysis. The colon was chosen as the site of investigation because of its fermentation capacity and the health implications. A 300-mg portion of fresh colonic luminal content was used for bacterial DNA extraction and another 300-mg portion for SCFA analysis.

Measurement of bacterial fermentation products. Fresh colonic luminal contents (300 mg) were analyzed for SCFAs, including acetate, propionate, and butyrate, by using gas chromatography with an Agilent 7890A system (Agilent Technologies) (14). Concentrations of biogenic amines in the colonic contents were measured with HPLC (15). Fecal sulfide concentration was determined by the methylene blue method as described previously (16). The OD_{665} value was recorded and the concentration of sulfide was calculated according to the standard curve.

Microbial DNA isolation and real-time qPCR analysis of the bacterial 16S ribosomal RNA gene. Total genomic DNA of bacteria in colonic content was extracted from 300 mg fresh colonic luminal content by using a bead-beating method and phenol-chloroform extraction (17). The DNA concentration was determined with a Nanodrop 2100 spectrophotometer (Thermo Fisher Scientific). Numbers of *Clostridiales* (*C. coccoides*, *C. leptum* cluster), *Lactobacillus*, *Bifidobacterium*, *Escherichia coli*, *Akkermansia muciniphila*, and sulfate-reducing bacteria (SRB) were quantified by using species-specific primers as listed in **Supplemental Table 2**. qPCR assay was performed on an ABI StepOne platform (Applied Biosystems) by using SYBR Premix Ex Taq dye (Takara). Standard curves were generated with 10-fold serial plasmid DNA containing the insert of a specific 16S ribosomal RNA (rRNA) gene purified from the respective target strains. Quantification of 16S rRNA gene copies in each sample was performed in triplicate, and the mean values were calculated. The data were expressed as log 16S rRNA gene copies per gram of colonic content.

High-throughput pyrosequencing analysis. After genomic DNA extraction and quantification, samples were prepared for amplification and sequencing at the Chinese National Human Genome Center (Shanghai, China) using the 454 GS FLX Titanium platform (Life Sciences). The V3–V4 region of the 16S rRNA gene was amplified using a universal forward primer (5′-TACGGRAGGCAGCAG-3′) and a reverse primer (5′-AGGGTATCTAATCCT-3′). The forward primer comprised the 454

[6] Abbreviations used: *Aebp1*, adipocyte enhancer binding protein 1; *Atp6v1b2*, ATPase, H transporting, lysosomal V1 subunit B2; *Bcl2l12*, BCL2-like 12 (proline rich); *Ccrl2*, chemokine (C-C motif) receptor-like 2; *Cry1*, cryptochrome 1; *Cx3cl1*, chemokine (C-X3-C motif) ligand 1; DEG, differentially expressed gene; *DsrA*, dissimilatory sulphite reductase A; *F8*, coagulation factor VIII; *Gpx7*, glutathione peroxidase 7; HPD, high-protein diet; IBD, inflammatory bowel disease; *Il2rg*, interleukin 2 receptor γ; *Itgb6*, integrin β6; *Magmas*, mitochondria-associated protein involved in granulocyte-macrophage colony-stimulating factor signal transduction; *Mgst1*, microsomal glutathione S-transferase 1; *Mx1*, myxovirus (influenza virus) resistance 1; *Ndufa1*, NADH dehydrogenase (ubiquinone) 1α subcomplex 1; *Nlrx1*, nucleotide-binding oligomerization domain-like receptor family member X1; NPD, normal-protein diet; OTU, operational taxonomic unit; OXPHOS, oxidative phosphorylation; *Parp1*, poly (ADP-ribose) polymerase 1; *Rarg*, retinoic acid receptor γ; *Ripk1*, receptor (tumor necrosis factor receptor superfamily)-interacting serine-threonine kinase 1; *Rps6ka3*, ribosomal protein S6 kinase polypeptide 3; *Smarcc1*, switch/sucrose non-fermenting related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1; SRB, sulfate-reducing bacteria; *St6gal1*, ST6 β-galactosamide α-2,6-sialyltranferase 1; *Trim32*, tripartite motif-containing 32; *Xpo5*, exportin 5.

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sequencing adapter A (5′-CCATCTCATCCCTGCGTGTCTCCGACG-ACT-3′), a sample barcode octamer, and an rRNA-specific sequence at the 5′ end. The reverse primer comprised the 454 sequencing adapter B (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3′) and the rRNA-specific sequence at the 5′ end. After being purified with Agencourt AMPure XP (Beckman), the PCR amplicons from different samples were pooled and sequenced on a 454 GS FLX Titanium platform.

Raw sequence data were trimmed, filtered, aligned, and classified using the mothur software package (version 1.32.0, Department of Microbiology and Immunology, The University of Michigan) (18). Sequences shorter than 200 bp, having one or more ambiguous base, or containing a homopolymer length ≥8 bp were removed from the data set. After trimming the primer, barcode, and chimeras, the unique sequences were identified and aligned against a high-quality 16S rRNA sequence from the Silva database (release 108) (19). After screening, filtering, and preclustering processes, gaps in each sequence were removed in all samples to reduce noise. Using the mean neighbor algorithm with a cutoff of 97% similarity, these sequences were clustered to operational taxonomic units (OTUs). Representative sequences from each OTU were taxonomically classified with a 90% confidence level by using the Ribosomal Database Project classifier (20). Representative sequences were also loaded into the National Center for Biotechnology Information Basic Local Alignment Search Tool website against the 16S rRNA sequence database (21). Bacterial diversity was assessed with rarefaction analysis, an abundance-based coverage estimator, a bias-corrected Chao richness estimator, Shannoneven (a Shannon index-based evenness), and the Shannon and Simpson diversity index (18). Principal coordinates analysis from mothur output was produced based on unweighted distances (18). The difference of sequence percentage between the NPD and HPD groups was evaluated with a nonparametric Mann-Whitney *U* test. The sequence information was submitted to the GenBank under accession number KP799013-KP8121147.

Colonic epithelial transcriptome profiling. Total RNA was isolated from colonic mucosa (100 mg) using the Trizol reagent (Invitrogen), and the quality and quantity of total RNA were determined by the Agilent 2100 Bioanalyser (Agilent Technologies) and Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Only RNA samples with a RNA integrity number ≥7.0 were included in the analysis. To balance the cost of replicates and the number of replicates necessary, we randomly selected 6 samples each from the NPD and HPD groups for microarray analysis by using the Agilent Genechip system (Agilent Technologies), which contains 28,282 probe sets (probe length: 60 mer, 4 × 44K format). cDNA synthesis, in vitro transcription, labeling, hybridization, and scanning were performed according to the standard procedures for one-color microarray analysis (Agilent Technologies), as described previously (22). Expression data were normalized through quantile normalization using the Robust Multichip Average algorithm. Array data were normalized to the global median signal intensity value, and values were log base 2 transformed. After data normalization and filtering, differentially expressed genes (DEGs) were defined by using a 2-fold cutoff. The microarray data have been submitted in the Minimum Information About a Microarray Experiment-compliant format to the National Center for Biotechnology Information Gene Expression Omnibus public database under the identifier GSE65862.

Real-time qPCR analysis of colonic epithelial genes. To confirm the gene expression of the microarray results, we conducted reverse transcription qPCR for 14 selected genes with primers listed in Supplemental Table 2. Reverse transcription was performed with 1 μg colonic RNA for first-strand cDNA synthesis with the PrimeScript RT reagent kit with gDNA Eraser (Takara) according to the manufacturer's instructions. All primer sets were validated to ensure efficient amplification of a single product before being used in assays. Each cDNA was analyzed in triplicate, and the mean threshold cycle was calculated. The results were normalized to the expression of the 18S rRNA gene, and relative expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis. Both parametric (Student's *t* test) and nonparametric (Mann-Whitney *U* test) methods were used to explore differences between treatments. Differences were considered significant at $P < 0.05$. Pairwise statistical analysis of DEGs was conducted by using the Student's *t* test with Benjamini-Hochberg multiple-testing correction (adjusted $P < 0.05$). An unweighted principal coordinates analysis was conducted by using mothur. Correlations between mucosal genes (DEGs from microarray analysis) with bacterial abundance (genus proportion from pyrosequencing analysis) or fermentation products were analyzed by using Spearman's ρ correlation analysis (XLStat software; Addinsoft). Correlation was considered significant when the absolute value of Spearman's rank correlation coefficient (Spearman's ρ) was >0.6 and statistically significant ($P < 0.05$).

Results

Growth performance and food intake. In this study, the HPD significantly reduced ($P < 0.01$) food intake (mean ± SEM: 13.9 ± 0.38 g/d compared with 17.5 ± 0.47 g/d) and body weight (mean ± SEM: 1.01 ± 0.16 g/d compared with 3.24 ± 0.22 g/d) compared with the NPD. The detailed data on growth performance were reported in supplemental material previously (23).

Colonic microbiota community. In total, 166,109 qualified bacterial 16S rRNA gene reads were obtained from 20 colon content samples after pyrosequencing and used for subsequent analysis. Species richness at the taxonomic level, as reflected by the abundance-based coverage estimator and Chao 1 index, was increased in the HPD group compared with the NPD group ($P < 0.01$, **Figure 1**B). The Shannon and Simpson diversity index and the community evenness did not differ between the NPD and HPD groups (Figure 1B). Moreover, OTU-based principal coordinates analysis showed distinct microbiota composition between the 2 groups (Figure 1C).

Among the bacterial groups, *Firmicutes* and *Bacteroidetes* were the 2 predominant phyla, contributing 48.6% and 40.6% of gut microbiota in the NPD group and 40.2% and 50.9% in the HPD group, respectively (Figure 1D). *Proteobacteria* and *Actinobacteria* constituted the next 2 most dominant phyla at 7.05% and 3.25% in NPD rats and 8.09% and 0.75% in HPD rats, respectively. The HPD significantly decreased the abundance of *Actinobacteria* and *Acidobacteria* ($P < 0.05$, Figure 1D) compared with the NPD group. At the genus level, *Barnesiella*, *Blautia*, *Prevotella*, *Coprococcus*, *Lawsonia*, *Bacteroides*, *Sporobacter*, *Parabacteroides*, and *Bifidobacterium* were the abundant genera (>1%) (**Figure 2**). Among the dominant genera, the abundance of *Lawsonia*, *Bacteroides*, and *Parabacteroides* was higher, whereas the abundance of *Sporobacter* and *Bifidobacterium* was lower in the colon of HPD rats compared with NPD rats (Figure 2). Interestingly, the HPD increased ($P < 0.05$) the abundance of *Escherichia/Shigella*, *Enterococcus*, and *Streptococcus* compared with the NPD, with a 54.9-fold increase in *Escherichia/Shigella* and a 31.3-fold increase in *Enterococcus* (Figure 2). In addition, the abundance of *Ruminococcus* and *Akkermansia* in HPD rats was significantly lower ($P < 0.05$) than that in NPD rats (Figure 2). The *Escherichia* to *Ruminococcus* ratio in the HPD was 385-fold ($P < 0.05$) higher than that in the NPD group (data not shown).

In total, 1832 OTUs with a detected read number >5 were recorded (**Supplemental Table 3**). Supplemental Table 3 shows all OTUs with read numbers >5, the number of reads for each OTU, and their percent abundance relative to the total sequences. Also, 999 OTUs (abundance >0.1% in at least one sample) were detected as predominant, with 845 OTUs shared between 2 groups, 82 unique in the NPD group and 72 unique in the HPD group. Among the 845 shared OTUs, 47 were significantly increased and 46 were significantly decreased in the HPD group compared with the NPD group (**Supplemental Table 4**). Among

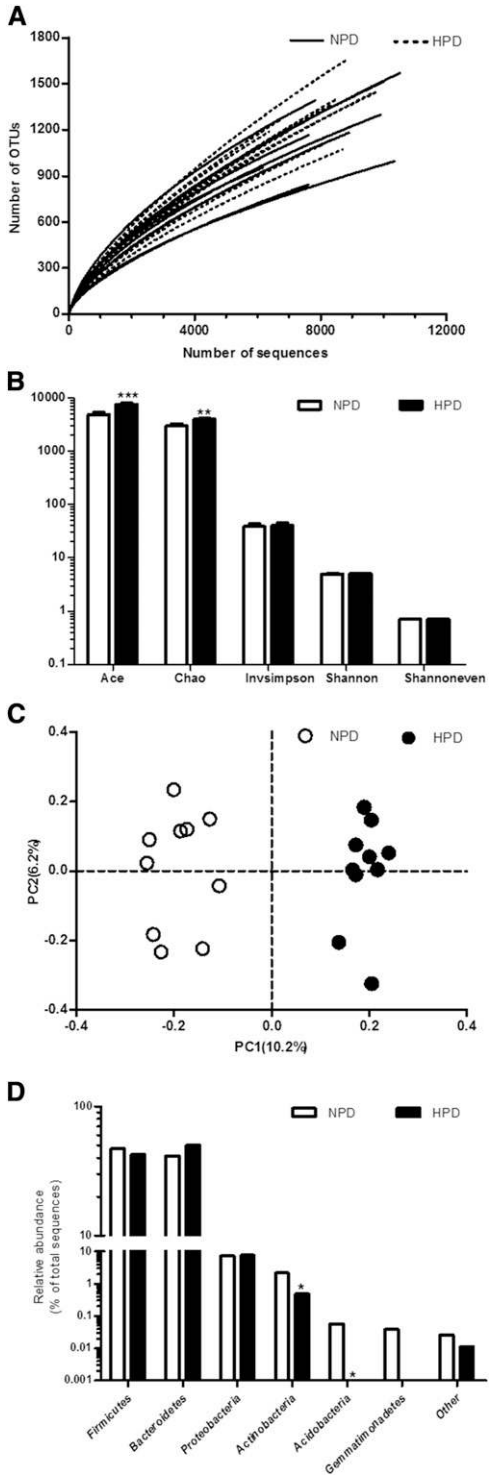

FIGURE 1 Pyrosequencing analysis of colonic microbiota of adult male Wistar rats fed NPDs and HPDs for 6 wk. (A) Rarefaction curves in the HPD and NPD groups. (B) Diversity indexes in the HPD and NPD groups. Values are means ± SEMs (n = 10). Asterisks indicate statistically significant difference from the NPD (Student's t test): $^{**}P < 0.01$, $^{***}P < 0.001$. (C) Principal coordinates analysis plots based on the relative abundance of OTUs at a 97% similarity level. (D) Phylum-level abundance of 16S rRNA gene sequences from the colon of NPD and HPD rats. Values are medians (n = 10). Asterisks indicate statistically significant difference from NPD (Mann-Whitney U test): $^{*}P < 0.05$. ACE, abundance-based coverage estimator; HPD, high-protein diet; NPD, normal-protein diet; OTU, operational taxonomic unit; PC1, principal coordinate 1; PC2, principal coordinate 2; rRNA, ribosomal RNA.

the OTUs, we identified those that were changed correspondingly with the affected genera by the HPD. Among the unique OTUs in the NPD group, one was closely related to *Ruminococcus bromii* (99% similarity) and one belonged to the genus *Roseburia* (Supplemental Table 4). Among those shared OTUs, many were higher in the colons of HPD rats than in those of NPD rats, with their closely related species as *E. coli/Shigella flexneri*, *Bacteroides vulgatus*, *Bacteroides fragilis*, *Lactobacillus murinus*, *Streptococcus hyointestinalis*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*, respectively. In addition, the abundance of several OTUs belonging to *Roseburia*, *Bifidobacterium* (*Bifidobacterium animalis*), and *Faecalibacterium* (*F. prausnitzii*) was lower in the colons of HPD rats than in those of NPD rats. The abundance of OTU0559 (*E. coli/S. flexneri*) was 54.9-fold greater ($P < 0.05$) in the HPD group than in the NPD group, whereas the abundance of OTU0416 (*F. prausnitzii*–like) was 3.92-fold lower ($P < 0.05$) in the HPD group than in the NPD group (Figure 3). The OTU related with *R. bromii* was solely detected in the NPD rats (Figure 3). These results suggest that the HPD significantly changed the colonic microbiota, with an increase in some potential pathogens and a decrease in generally beneficial bacteria in rats.

qPCR on colonic microbiota showed no significant differences in the phyla *Firmicutes* and *Bacteroidetes* between the 2

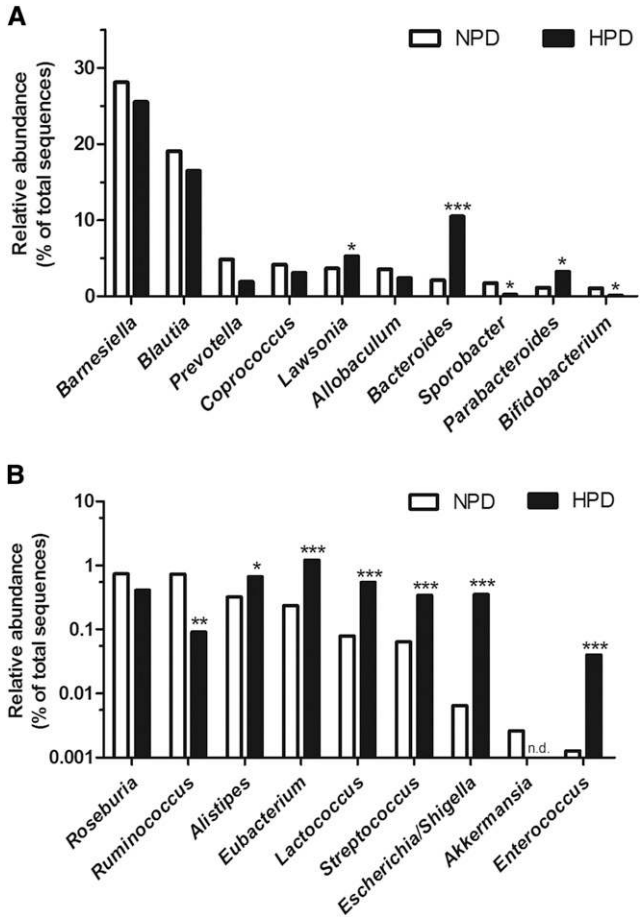

FIGURE 2 Effect of HPDs on the microbial communities at the genus level in colonic contents of adult male Wistar rats compared with rats fed NPDs for 6 wk. The predominant genus (A) and the significantly changed genus (B) are shown. Values are medians (n = 10). Asterisks indicate statistically significant difference from NPD (Mann-Whitney U test): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. HPD, high-protein diet; n.d., zero sequence detected in the HPD group; NPD, normal-protein diet.

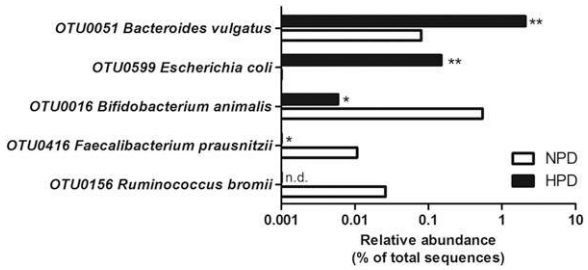

FIGURE 3 Effect of HPDs on the representative phylotypes in colonic contents of adult male Wistar rats compared with rats fed NPDs for 6 wk. Values are medians (n = 10). Asterisks indicate statistically significant difference from NPD (Mann-Whitney U test): $^*P < 0.05$, $^{**}P < 0.01$. HPD, high-protein diet; NPD, normal-protein diet; n.d., zero sequence detected in the HPD group; OTU, operational taxonomic unit.

groups (**Supplemental Figure 1**). Compared with the NPD, the HPD significantly decreased ($P < 0.05$) the number of the genus *Akkermansia* and tended to reduce the number of *C. leptum* and *C. coccoides* clusters (P = 0.10 and 0.05, respectively), while increasing ($P < 0.05$) the number of *Lactobacillus*, *E. coli*, and SRB.

Microbial metabolism in the colon. The concentrations of SCFAs, amines, and sulfides were measured as indicators of microbial fermentation. The concentration of butyrate was significantly higher ($P < 0.05$) in the HPD group than in the NPD group. The concentrations of acetate and propionate tended to be lower (P = 0.07 and 0.09, respectively) in the colon of the HPD rats than NPD rats. Cadaverine, putrescine, tryptamine, methylamine, and spermine were the major biogenic amines in the colon. Among them, methylamine, cadaverine, and spermine increased ($P < 0.05$) in the HPD group compared with the NPD group (**Figure 4**), but there was no significant difference in tyramine, tryptamine, spermidine, or its precursor, putrescine, in the HPD group compared with the NPD group. Moreover, the significant correlations ($P < 0.05$) were observed between the colonic cadaverine and *Enterococcus*, *Streptococcus*, and *Escherichia/Shigella*, with a Spearman's ρ of 0.81 (95% CI: 0.37, 0.95), 0.78 (95% CI: 0.30, 0.94), and 0.75 (95% CI: 0.23, 0.94), respectively. Compared with the NPD, the HPD significantly increased fecal sulfide concentrations (0.85 compared with 0.18 mg feces/g, $P < 0.01$). These results indicate that the HPD significantly decreased the production of the beneficial compound butyrate while increasing protein-fermenting products in rats.

Transcriptional response of the colon mucosa. Among the 30,366 rat genes investigated, a total of 8992 genes were expressed in the colon, and 181 genes were significantly differentially expressed (>2-fold change) between the 2 groups, with 88 upregulated and 93 downregulated in HPD rats compared with NPD rats (**Supplemental Table 5**). Because this study focused on the genes involved in the microbiota-related immune response and metabolic processes, 14 genes were selected for relative PCR quantification. The quantifications of the 14 selected genes, including encoding microsomal glutathione S-transferase 1 (*Mgst1*), encoding glutathione peroxidase 7 (*Gpx7*), and BCL2-like 12 (proline rich) (*Bcl2l12*) [encoding BCL2-like 12 (proline rich)] (**Supplemental Figure 2**), further confirmed the changes of the colonic transcriptome in response to the HPD.

A number of genes involved in the microbiota-related immune responses and metabolic processes were quantified. The genes encoding chemokine (C-X3-C motif) ligand 1 (CX3CL1) and chemokine (C-C motif) receptor-like 2 (CCRL2) that are involved in chemotaxis were also upregulated ($P < 0.05$) in the HPD group compared with the NPD group (**Table 1**). The genes involved in cell adhesion (an essential step for chemotaxis) were upregulated ($P < 0.05$) in the HPD group compared with the NPD group, including encoding coagulation factor VIII (*F8*) and encoding integrin β6 (*Itgb6*). The TNF-α signal process was upregulated ($P < 0.05$) in the HPD group compared with the NPD group, as inferred from the upregulation of encoding tripartite motif-containing 32 (*Trim32*), [encoding receptor (tumor necrosis factor receptor superfamily)-interacting serine-threonine kinase 1 (*Ripk1*)], and [encoding poly (ADP-ribose) polymerase 1 (*Parp1*)]. The genes *Mgst1* and *Gpx7* involved in the glutathione S-transferase pathway were upregulated ($P < 0.05$) in the HPD group compared with the NPD group. These results indicate the HPD upregulated genes involved in disease pathogenesis in rats.

Compared with the NPD, the HPD downregulated ($P < 0.05$) the expression of genes involved in innate immunity, including encoding interleukin 2 receptor γ (*Il2rg*), encoding mitochondria-associated protein involved in granulocyte-macrophage colony-stimulating factor signal transduction (*Magmas*), encoding myxovirus resistance 1 (*Mx1*), and encoding retinoic acid receptor γ (*Rarg*). The oxidative phosphorylation (OXPHOS) seemed to be suppressed, as inferred from the downregulation ($P < 0.05$) of encoding ATPase, H transporting, lysosomal V1 subunit B2 (*Atp6v1b2*), encoding cryptochrome 1 (*Cry1*), and [encoding NADH dehydrogenase (ubiquinone) 1α subcomplex 1 (*Ndufa1*)] in the HPD group compared with the NPD group. The colonic gene expression also disclosed the downregulation ($P < 0.05$) of genes involved in O-linked glycosylation of mucin in the HPD group compared with the NPD group. These results indicate the downregulation of genes involved in immunoprotection in the HPD rats.

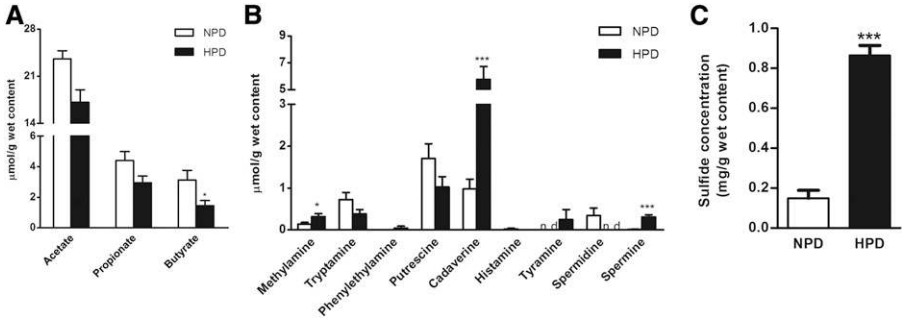

FIGURE 4 Effect of HPDs on the concentrations of SCFAs (A), biogenic amines (B), and fecal sulfide (C) of adult male Wistar rats compared with rats fed NPDs for 6 wk. Values are means ± SEMs (n = 10). Asterisks indicate statistically significant difference from NPD (Student's t test): $^*P < 0.05$, $^{***}P < 0.001$. The limits of detection for tyramine and spermine are 0.5 and 0.1 μmol/L, respectively. HPD, high-protein diet; n.d., not detected; NPD, normal-protein diet.

TABLE 1 Differentially expressed genes in the colon of adult male Wistar rats fed HPDs compared with rats fed NPDs for 6 wk[1]

Gene symbol	RefSeq	Description	NPD[2]	HPD[2]	Fold change[3]	FDR *P* value
Glutathione metabolism						
Gpx7	NM_001106673	Glutathione peroxidase 7	2.65	3.96	1.31	0.036
Mgst1	NM_134349	Microsomal glutathione S-transferase 1	0.53	2.74	2.21	0.037
Rps6ka3	NM_001192004	Ribosomal protein S6 kinase polypeptide 3	−2.6	0.04	2.64	0.026
Chemotaxis: Proinflammatory cytokines						
Cx3cl1	NM_134455	Chemokine (C-X3-C motif) ligand 1	−5.38	−1.76	3.62	0.035
Ccrl2	NM_001108191	Chemokine (C-C motif) receptor-like 2	2.75	3.87	1.12	0.049
Aebp1	NM_001100970	AE binding protein 1	−4.52	−0.16	4.36	0.033
Col12a1	U57362	Collagen, type XII, α1	−4.80	−0.95	3.86	0.038
Itgb6	NM_001004263	Integrin β6	−1.82	1.59	3.40	0.021
F8	NM_183331	Coagulation factor VIII, procoagulant component	1.85	4.68	2.83	0.006
Antigen presentation						
LOC498276	NM_001135992	Fc γ receptor IIβ	−5.01	−0.65	4.36	0.002
Nlrx1	NM_001025010	nucleotide-binding oligomerization domain-like receptor family member ×1	1.38	4.00	2.62	0.002
TNF-α signaling pathway						
Trim32	NM_001012103	Tripartite motif-containing 32	−2.47	1.41	3.88	0.030
Ripk1	NM_001107350	Receptor (TNFRSF)–interacting serine-threonine kinase 1	−3.48	0.12	3.60	0.044
Parp1	NM_013063	Poly (ADP-ribose) polymerase 1	1.89	4.53	2.64	0.009
Smarcc1	NM_001106861	SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily c, member 1	1.39	3.67	2.28	0.000
Apoptosis						
Bcl2l2	NM_021850	Bcl2-like 2	−0.30	2.29	2.59	0.006
Cidea	NM_001170467	Cell death–inducing DFFA-like effector a	−1.32	2.29	3.62	0.018
Xpo5	NM_001108789	Exportin 5	2.93	4.33	1.40	0.002
Siva1	NM_001100982	SIVA1, apoptosis-inducing factor	2.89	4.00	1.11	0.048
Oxidative phosphorylation						
Ndufa1	NM_001108813	NADH dehydrogenase (ubiquinone) 1α subcomplex, 1	4.24	2.80	−1.44	0.017
Atpif1	NM_012915	ATPase inhibitory factor 1	1.50	−0.61	−2.11	0.048
Cry1	NM_198750	Cryptochrome 1 (photolyase-like)	−1.39	−4.08	−2.69	0.045
Atp6v1b2	NM_057213	ATPase, H transporting, lysosomal V1 subunit B2	0.41	−3.00	−3.41	0.024
O-linked glycosylation of mucins						
B3gnt2	NM_001107240	UDP-GlcNAc:βGal β-1,3-N-acetylglucosaminyltransferase 2	1.81	−2.78	−4.59	0.011
St6gal1	NM_147205	ST6 β-galactosamide α-2,6-sialyltranferase 1	−1.61	−4.73	−3.12	0.047
Pofut2	NM_001107621	Protein O-fucosyltransferase 2	−0.40	−4.73	−4.33	0.004
Alg11	NM_001108401	Asparagine-linked glycosylation 11, α-1,2-mannosyltransferase homolog	1.27	−1.41	−2.68	0.026
Innate immune						
Il2rg	NM_080889	Interleukin 2 receptor, γ	1.19	−3.29	−4.49	0.008
Magmas	NM_001100136	Mitochondria-associated protein involved in granulocyte-macrophage colony-stimulating factor signal transduction	2.11	−2.02	−4.13	0.016
Mx1	NM_173096	Myxovirus (influenza virus) resistance 1	2.15	−1.47	−3.62	0.043
Rarg	NM_001135249	Retinoic acid receptor γ	2.54	0.86	−1.68	0.004
Lipid storage						
Cry1	NM_198750	Cryptochrome 1 (photolyase-like)	−1.39	−4.08	−2.69	0.045
Cd36	NM_031561	CD36 molecule (thrombospondin receptor)	6.07	4.20	−1.87	0.023
Scd1	NM_139192	Stearoyl–coenzyme A desaturase 1	−4.80	−1.20	3.60	0.049
Olfactory transduction						
Calm1	NM_031969	Calmodulin 1	0.36	−2.79	−3.15	0.043
Gnal	NM_001191836	Guanine nucleotide binding protein, α stimulating, olfactory type	0.64	−3.12	−3.76	0.007

[1] Enriched pathways are derived from Ingenuity Pathway Analysis, WebGestalt, and Enrichr enrichment analysis. AE, adipocyte enhancer; DFFA, DNA fragmentation factor-α; FDR, false discovery rate; HPD, high-protein diet; NLR, nucleotide-binding oligomerization domain-like receptor; NPD, normal-protein diet; RefSeq, reference sequence; SWI/SNF, switch/sucrose non-fermenting; TNFRSF, tumor necrosis factor receptor superfamily.
[2] Values represent fluorescent intensity of probe set and are presented in $\log_2$ space. Values are means, $n = 6$.
[3] Mean $\log_2$ fold-change of the signal intensity of the HPD group compared with the NPD group. Positive values represent upregulation, whereas negative values indicate downregulation.

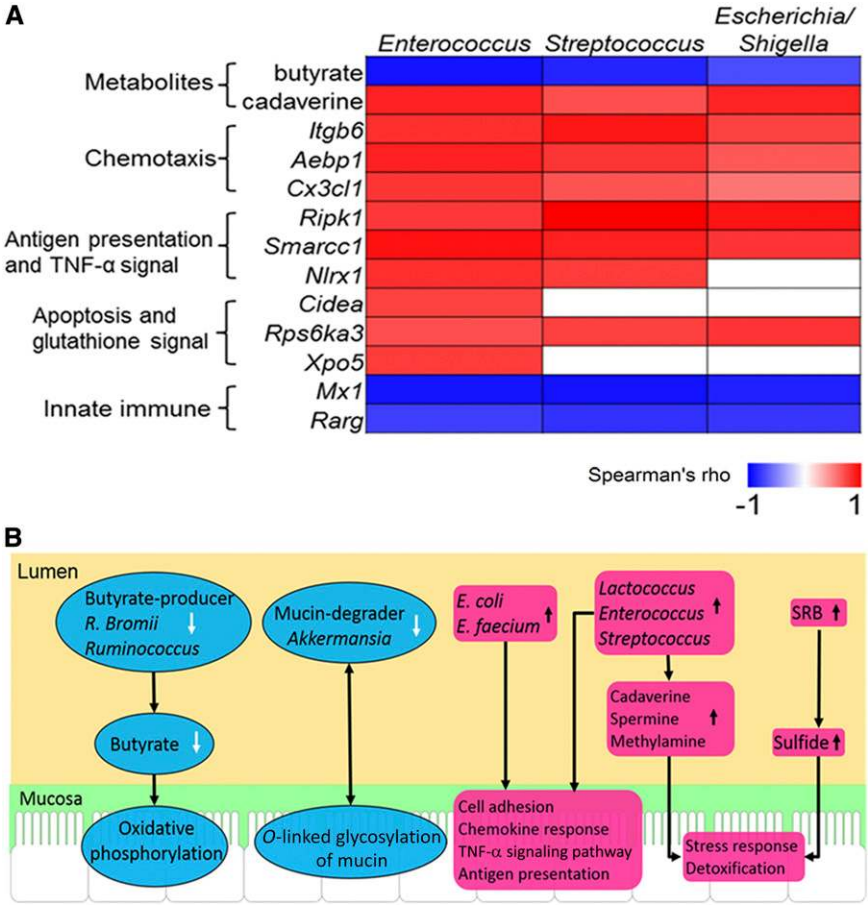

FIGURE 5 (A) Spearman's rank correlation between abundance of *Enterococcus*, *Streptococcus*, *Escherichia/Shigella*, and metabolites and epithelial gene expression in the colon of adult male Wistar rats fed normal-protein diets and high-protein diets for 6 wk. The intensity of the colors represents the degree of association. (B) Proposed function model of high-protein diets. Items with a black up-arrow indicate the increased bacteria, metabolites, or functions in the high-protein diet group compared with the normal-protein diet, whereas those with a white down-arrow indicate the decreased ones in the high-protein diet group. *Aebp1*, adipocyte enhancer binding protein 1; *Cidea*, cell death–inducing DNA fragmentation factor-α-like effector a; *Cx3cl1*, chemokine (C-X3-C motif) ligand 1; *Itgb6*, integrin β6; *Mx1*, myxovirus (influenza virus) resistance 1; *Nlrx1*, nucleotide-binding oligomerization domain-like receptor family member ×1; *Rarg*, retinoic acid receptor γ; *Ripk1*, receptor (tumor necrosis factor receptor superfamily)–interacting serine-threonine kinase 1; *Rps6ka3*, ribosomal protein S6 kinase polypeptide 3; *Smarcc1*, switch/sucrose non-fermenting related, matrix associated, actin-dependent regulator of chromatin, subfamily c, member 1; SRB, sulfate-reducing bacteria; *Xpo5*, exportin 5.

Correlation between mucosal genes with bacteria or bacterial fermentation products. The results showed that the abundance of *Enterococcus*, *Streptococcus*, and *Escherichia/Shigella* was negatively correlated with the concentration of butyrate, a beneficial microbial product. However, the abundance of *Enterococcus*, *Streptococcus*, and *Escherichia/Shigella* was positively correlated with the concentration of cadaverine, a harmful product from protein fermentation (**Figure 5**A). Furthermore, the abundance of *Enterococcus*, *Streptococcus*, and *Escherichia/Shigella* showed positive correlations with a set of upregulated genes involved in chemotaxis [chemokine (C-X3-C motif) ligand 1 (*Cx3cl1*), adipocyte enhancer binding protein 1 (*Aebp1*), *Itgb6*], the TNF-α signaling process [*Ripk1*, switch/sucrose non-fermenting related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1 (*Smarcc1*)], and the glutathione S-transferase pathway (*Rps6ka3*). Alternatively, the abundance of *Enterococcus*, *Streptococcus*, and *Escherichia/Shigella* were negatively correlated with the downregulated genes involved in innate immunity (*Mx1*, *Rarg*) (Figure 5A). The abundance of *Akkermansia* correlated positively with the gene ST6 β-galactosamide α-2,6-sialyltranferase 1 (*St6gal1*), which is involved in *O*-linked glycosylation of mucin (Spearman's ρ = 0.72; 95% CI: 0.17, 0.93; $P < 0.01$), suggesting a relation between *Akkermansia* and mucin synthesis. The concentration of sulfide correlated positively with *Mgst1*, which is involved in oxidative stress (Spearman's ρ = 0.70; 95% CI: 0.13, 0.92; $P < 0.05$). These results suggest that the changes in the colonic microbiota are correlated with alterations of metabolites and epithelial gene expression in rats.

Discussion

Studies have investigated the effect of HPDs on gut bacterial species and their metabolites with fecal material (4, 5) and on certain bacterial species and gene expression in the rat colon (6, 12). For the first time, to our knowledge, the present study combined the microbiome and transcriptome analysis to explore the impact of HPDs on colonic health risks. Our results show that the HPD led to an imbalanced colon microbiome and metabolic profile and changed the epithelial function of the colon toward the risk of colonic disease in rats.

The HPD changed the colonic microbiota and their metabolites. The present study with pyrosequencing led to a marked finding that the HPD caused an imbalanced colonic microbiome by increasing the abundance of many potentially opportunistic pathogens while reducing the abundance of potentially beneficial species. Bacterial species such as *E. coli*, *Enterococcus* spp., and *Streptococcus* spp. were markedly increased in their abundance in HPD rats (Figure 2). The increases in *E. coli* and *B. vulgatus* have been shown to be involved in the development of Crohn disease (24). Some species within the genera *Escherichia/Shigella*, *Enterococcus*, and *Streptococcus*, including *E. coli* (25) and *E. faecium* (26), are known as opportunistic pathogens and are associated with colitis or Crohn disease. The abundance of *Escherichia/Shigella*, *Enterococcus*, and *Streptococcus* was higher in the feces of patients with colorectal cancer compared with healthy volunteers (27). These results suggest that the HPD increased the bacteria associated with increased colonic disease. On the other

hand, the HPD decreased some butyrate-producing bacteria such as *R. bromii* and *F. prausnitzii* in the colon (Figure 3). *R. bromii* and *F. prausnitzii*, together with butyrate, could benefit colonic homeostasis by promoting epithelial energy metabolism and modulating immune development (3). Interestingly, we found a significant increase in the *Escherichia* to *Ruminococcus* ratio in rats fed the HPD. Some *Ruminococcus* bacteria are major acetate and butyrate producers (28), which can benefit the healthiness of the gut. The shift in the *Escherichia* to *Ruminococcus* ratio indicated a remarkable alteration of the microbiota community in rats fed the HPD. Moreover, *F. prausnitzii* was reported to have an anti-inflammatory action against Crohn disease (29). A previous study has also shown that an HPD reduced the numbers of *C. coccoides*, *C. leptum* groups, and *F. prausnitzii* in the colonic lumen of rats (6). In the present study, another important finding was that the HPD reduced the abundance of the mucin degrader *Akkermansia*. *A. muciniphila* is reported to be a health-promoting bacterium and potential probiotic (30). A low number of *A. muciniphila* has been reported in elderly patients with Crohn disease or ulcerative colitis (31). Taken together, these findings suggest that the HPD impaired the balance of the colonic microbiota by increasing some disease-associated bacteria while reducing potentially beneficial bacteria.

Our study further demonstrated that the change in colonic microbiota was paralleled with altered microbial metabolism. The HPD reduced the concentration of butyrate in the colon (Figure 4). Butyrate is a major energy source for colonocytes (32). The energy metabolism via OXPHOS in the mitochondria is crucial for cellular processes. The downregulation of genes involved in mitochondrial OXPHOS, which may be associated with the reduction of butyrate, may reflect a detrimental effect of the HPD on mitochondrial function. Furthermore, butyrate exerts an anti-inflammatory function and inhibits tumorigenesis (33). The low butyrate production under the HPD may also indicate a decrease in immunity. Butyrate could also be produced from glutamate and lysine (34). In the present study, the HPD increased the concentration of cadaverine derived from lysine decarboxylation, suggesting an increased transformation of lysine toward cadaverine production rather than butyrate production. High amounts of cadaverine are toxic, because they induce oxidative stress and DNA damage (35), which increases the tumorigenesis risk. The HPD also increased sulfide significantly together with the increased SRBs [as inferred from dissimilatory sulphite reductase A (*DsrA*) gene copies] (Figure 4, Supplemental Figure 1). Sulfide may be detrimental to the colon. In vitro studies have shown that sulfide at a concentration as low as 250 μmol/L was able to cause significant genomic DNA damage in cultured Chinese hamster ovary cells (36). HPDs could increase colonic DNA damage with an increased carcinogenesis risk in rats (37). These reports and our results indicate the potential role of sulfide in regulating colonic health. Increased sulfide has been observed in patients with colorectal cancer compared with healthy individuals (38). Sulfide also inhibits butyrate oxidation by inhibiting the cytochrome oxidase in mitochondria (39), which could lead to a reduced utilization of butyrate for colonic cell growth or renewal. Thus, the increased load of potential detrimental metabolites, including amines (cadaverine, spermine) and sulfide, could add weight to the detrimental influence of the HPD due to the decrease of butyrate in the colon.

We also found that the cadaverine concentration was positively correlated with the abundance of *Enterococcus*, *Streptococcus*, and *Escherichia/Shigella*. Bacteria within these genera are capable of producing cadaverine through decarboxylation of amino acids and subsequently using the polyamines to increase their virulence (40). Collectively, these findings suggest that the HPD led to a marked shift of microbial metabolites toward the production of unhealthy products in the colon, indicating a potential risk of colonic disease. Both the changes in the microbiome and metabolites favored a disadvantageous state in the colon of rats fed the HPD.

The HPD affected the epithelial response involved in colonic health. Our transcriptomic analysis showed upregulations of genes involved in colonic diseases such as inflammatory bowel disease (IBD) in HPD rats. The present study identified the upregulation of IBD-related genes *Sbno2* and *Fcgr2b* in HPD rats. The human orthologs of genes *Sbno2* and *Fcgr2b* have been shown to be associated with IBD, as revealed in genome-wide association studies (41). Genes *Sbno2* and *Fcgr2b* may serve as key players for regulating colonic health in response to the HPD. The HPD also markedly elevated genes involved in chemotaxis and oxidative stress in the colon (Table 1). The HPD upregulated genes *Cx3cl1* and chemokine (C-C motif) receptor-like 2 (*Ccrl2*), which are involved in the chemokine responses. Elevations of both the chemokine response and oxidative stress have also been observed in patients with IBD (2). Gene expression of *Cx3cl1* and *Ccrl2* was upregulated in patients with IBD (42). For oxidative stress changes, we found that genes *Gpx7* and *Mgst1* were upregulated in the HPD rats (Table 1). Oxidative stress is a key step in IBD pathogenesis. Therefore, the upregulation of genes involved in the chemokine response and oxidative stress in HPD rats may be involved in the increased colonic disease risk.

The HPD downregulated genes involved in the innate immune response in the colon as well as *Mx1* gene expression. *Mx1* encodes the interferon-induced dynamin-like GTPase involved in the innate immune defense (43). *Mx1* is essential for epithelial homeostasis, as diverse enteric viruses and bacteriophages inhabit the gut (44). Thus, the downregulation of *Mx1* suggests there is an alteration in the immune response in the colonic epithelium in HPD rats.

Colonic microbiota change is associated with altered intestinal gene expression. A novel finding of this study is that the colonic immune response was closely related with colonic microbiota. For example, the abundance of *Enterococcus*, *Streptococcus*, and *Escherichia/Shigella* was positively correlated with genes involved in chemotaxis but negatively correlated with genes involved in innate immunity (Figure 5). *Escherichia/Shigella* can serve as extracellular triggers inducing the cellular immune response, including antigen presentation, cell adhesion, and chemokine response (45). Thus, the observed upregulation of chemotaxis-related genes in the present study may be attributed to the increased abundance of *Escherichia/Shigella* species. Therefore, *Enterococcus*, *Streptococcus*, and *Escherichia/Shigella* may be important bacterial groups mediating the detrimental effects of the HPD.

The HPD increased the abundance of *Lactobacillus* species, such as *L. delbrueckii* subsp. *bulgaricus* and *L. murinus* (Supplemental Table 4). These *Lactobacillus* species have a strong ability to use amino acids (46), which could lead to the increased production of biogenic amines such as cadaverine. The increased protein content (casein source) in the HPD may be involved in the increase of *Lactobacillus* species. Although these *Lactobacillus* species may be potential probiotics, their immunologic role in the colon of HPD rats remains unknown. According to the results from epithelial gene expression, we have not observed the change of genes involved in the anti-inflammatory responses

that may be associated with *Lactobacillus* probiotics. Alternatively, we observed the upregulation of genes involved in chemotaxis, a proinflammatory process. These responses may result from potential pathogens or harmful products such as cadaverine. Collectively, the epithelial response to the microbiota tended to be affected more by potential pathogens than by potential probiotics.

Mucin, which is decorated with oligosaccharides linked via *O*-glycosidic bonds, provides the major nitrogen source for *A. muciniphila* (47). The positive correlation between *Akkermansia* abundance and *St6gal1* gene expression indicated the potential causal relation between the decrease in *Akkermansia* and the downregulation of glycan synthesis.

Diet, colonic microbiota, and colon health. In the current study, our collective findings suggested that the HPD could increase the risk of colonic disease through changes in both colonic functions as well as the microbiota and its metabolism. On the basis of these findings, we proposed a model of actions of the HPD impact on colonic health as outlined in Figure 5. The HPD changed the colonic microbiota with an increase in disease-associated bacteria and a decrease in potentially beneficial bacteria. These changes corresponded to the alteration of metabolic profiles and epithelial gene expression in the colon, which suggested a perturbation of colonic homeostasis. The reduction of butyrate may lead to the downregulation of intracellular OXPHOS. Furthermore, the evident reduction in the abundance of *Akkermansia* may be associated with the downregulation of the *O*-linked glycosylation of mucin. On the other hand, the increase in the abundance of some potentially disease-associated bacteria (*Escherichia*, *Enterococcus*, *Streptococcus*, and SRB) may result in the elevation of potentially harmful microbial metabolites such as cadaverine, spermine, and sulfide. These detrimental metabolites could lead to the upregulation of the stress response and detoxification process. In addition, a higher abundance of the potentially harmful bacteria *E. coli* and *E. faecium* may directly modulate mucosal cell adhesion, chemokine response, the TNF-α signaling pathway, and innate immunity, which still needs further demonstration. Our findings that the HPD led to an imbalanced colon microbiota, with the emerging production of harmful microbial metabolites and altered epithelial function in the colon, suggest that the HPD may elevate the colonic disease risk in rats.

In conclusion, this study, using an integrated approach combining microbe pyrosequencing and metabolic profiling and transcriptomics in a rat model, demonstrated that the HPD altered colonic gene expression, the microbiota, and microbial metabolic profiles. The HPD increased opportunistic pathogens while reducing the abundance of butyrate-producing bacteria and mucin degraders; there was also an increase in harmful microbial metabolites cadaverine, spermine, and sulfide and a decrease in butyrate. The HPD resulted in the transcriptional upregulation of genes involved in disease pathogenesis and downregulation of genes involved in immune protection. The results support our hypothesis that an HPD could increase the colonic disease risk in rats.

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