Dietary butyrate suppresses inflammation through modulating gut microbiota in high-fat diet-fed mice
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Dietary butyrate suppresses inflammation through modulating gut microbiota in high-fat diet-fed mice

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One sentence summary: Butyrate intervention suppressed proinflammatory cytokines through regulating TLR4 signaling pathways, SCFAs-producing bacteria and endotoxin-secreting bacteria, as well as levels of SCFAs and endotoxin.

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ABSTRACT

Butyrate, a key metabolite fermented by gut microbiota mainly from undigested carbohydrates such as dietary fibers is widely used as feed additive. However, mechanisms of its contributions in maintaining host health are relatively poorly revealed. The aim of this study was to investigate how butyrate impacts gut microbiota and immunity response in high-fat diet-fed mice. Gut microbial analysis exhibited that butyrate intervention increased short-chain fatty acids (SCFAs)-producing bacteria and decreased pathogenic bacteria, such as endotoxin-secreting bacteria. Our result also demonstrated that butyrate intervention enhanced fecal SCFAs concentrations, and inhibited endotoxin levels in feces and serum. Correlation analysis indicated positive relation between endotoxin level and Desulfovibrionaceae abundance. Furthermore, butyrate intervention inhibited expressions of IL-1β, IL-6 and MCP1/CCL2 in liver, as well as TLR4 in adipose tissue. Apart from inhibiting expressions of proinflammatory cytokines, butyrate exerted anti-inflammation effect through selectively modulating gut microbiota, such as increasing SCFAs-producing bacteria and decreasing endotoxin-secreting bacteria, as well as via regulating levels of microbiota-dependent metabolites and components, such as SCFAs and endotoxin.

Keywords: butyrate; gut microbiota; inflammation; endotoxin; SCFAs

INTRODUCTION

Gut microbiota has been shown to be involved in many important physiological processes, such as immune response, host development and metabolism. The hosts and gut microbiota is mutualistic symbiosis, and perturbation of the microbiome was related to several diseases, such as metabolic syndrome and inflammatory bowel disease (Sun et al. 2018; Wang et al. 2019). It has been reported that ~10% of metabolites found in mammalian blood were produced by bacteria (Wikoff et al. 2009). These microbiota-derived metabolites executed diverse functional roles in the host, such as short-chain fatty acids (SCFAs). SCFAs, mainly acetate, propionate and butyrate, are a major class of metabolites produced by the gut microbiota from undigested complex dietary carbohydrates. Moreover, sodium...
butyrate is a common component found in dietary foods, such as cheese and butter. Butyrate plays important physiology roles in host, such as maintaining balance of body electrolytes, providing energy to host and supplying nutrition to intestinal epithelial cells. However, mechanisms of its contributions in maintaining host health are relatively poorly revealed. Nowadays, some evidence supported the correlations between butyrate and specific microorganism, especially its roles in inhibiting pathogenic bacteria. For example, Vibrio sp. counts in the intestine of Litoperaeus vannamei was decreased after butyrate supplementation (da Silva et al. 2016). More recently, the proportions of Proteobacteria and Lachnospiraceae were enhanced and the proportion of Clostridiaceae was decreased after butyrate intervention in mice infected with Citrobacter rodentum (Jiminez et al. 2017).

Inflammation, which was associated with synthesis and secretion of endogenous cytokines or chemokines, accounts for important role in innate immunity (Rogero and Calder 2018). Enhancement expression of some cytokines, including interleukin-1β (IL-1β) and IL-6, accelerated progression of multiple disease, such as obesity-associated disease, metabolic syndrome and some types of cancer (Cox, West and Cripps 2015). It is well known that low-grade inflammation and disturbances of the gut microbiota can be induced by high-fat diet (HFD). Reports documented a potential role for butyrate in inflammation modulation. For example, butyrate addition decreased inflammation in fish with high parasite infection levels (Piazzon et al. 2017). Furthermore, butyrate may be beneficial in attenuating endotoxin-induced intestinal inflammation in pig (Melo et al. 2016). Butyrate thus is extensively used in commercial feed additive. Therefore, knowledge of the effects of butyrate on gut microbiota and immune system is of practical importance. The objective of this work was to evaluate how butyrate impacts gut microbial ecology and whether these microbial changes affect immunity response in high-fat diet-fed mice.

MATERIALS AND METHODS

Chemicals and reagents
Sodium butyrate (PharmaGrade, USP/NF, manufactured under appropriate GMP controls for pharma or biopharmaceutical production) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals and experimental design
All the experiments in this study were approved by the Animal Care and Use Committee of Binzhou Medical University (BMU No. 20100701-1). All C57BL/6 J male mice aged six-week-old were obtained from Pengyue Laboratory Animal Company (Jinan, China). Mice were acclimated for one week under controlled temperature at 23 ± 1°C, 40–70% relative humidity, 12/12 h light/dark cycle, with water and diet were applied ad libitum. Then mice were randomly divided into three groups (n = 5 per group): normal chew diet (NCD) group, high-fat diet group and high-fat diet with butyrate (HB) group. Composition of the NCD and HFD was available in Supplementary File 1 (Supporting Information). The additive amounted sodium butyrate was 1% (w/w). Fresh fecal samples were collected at 0 weeks and 8 weeks, and were maintained at −80°C for gut microbiota, SCFAs and endotoxin analysis. All mice were euthanized under deep anesthesia by intraperitoneal injection of sodium pentobarbital (200 mg kg⁻¹) after a 12 h fasting at the end of the experiment. Blood was harvested and centrifuged at 3500 rpm for 10 min at 4°C, and the serum was kept at −20°C for endotoxin analysis.

| Name          | Sequence(5’-3’) |
|---------------|----------------|
| IL-1β-F       | TCCAGGATGAGCAGATGAGCAC |
| IL-1β-R       | GAACGTCAACACACAGCAGTTA |
| IL-6-F        | CCACCTCAGAACGAGGCTTA |
| IL-6-R        | CCCAGTTGAGTACCCATCATTTT |
| MCP1/CCL2-F   | AGGCAGCAGGTGGCTCAAGA |
| MCP1/CCL2-R   | GTGCCGAGACCCCTAGGCAAGA |
| TLR4-F        | GGCCTAAACCCAGCTCTGTTCG |
| TLR4-R        | CTTCCTGCCGGAATGTCCTCA |
| Gapdh-F       | TGTTGGCCTGCTGGAAGTCTGA |
| Gapdh-R       | TTGCTCCTTGAAGTGCAGGAG |

Liver and adipose tissues were dissected and stored at −80°C for real-time polymerase chain reaction (RT-PCR) analysis.

Fecal DNA extraction and 16S rRNA sequencing
QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract total microbial DNA from fecal samples. The V4-V5 hypervariable 16S rRNA region was amplified from total DNA amplified used specific primer (515F 5’-GTGCCAGCMGCCGCGGTAA-3’ and 926R 5’-CCGYCAATTYMTTRAGTTT-3’) with the barcode, while PCR amplification was carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA). The PCR products were detected with 2% (w/v) agarose gels electrophoresis. Then the amplicon between 400 and 450 bp were purified by Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). 16S rRNA gene library was generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, USA). The 16S sequences were submitted to NCBI Sequence Read Archive (SRA) under accession number PRJNA509974.

Bioinformatic analysis
The raw tags obtained were quality-filtered by QIIME (Version 1.7.0). Sequences with more than 97% similarity were assigned to the same operational taxonomic units (OTUs) by Uparse (Version 7.0.1001) software. OTU abundances were normalized based on the sample with the least sequences. β diversity was calculated by QIIME software (Version 1.7.0) according to the normalized sequences. Principal component analysis (PCA) was performed with the FactoMineR package and ggplot2 package in R software (Version 2.15.3).

RNA isolation and RT-PCR
Total RNA was extracted from liver and adipose tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA) reverse-transcribed to cDNA using the reverse transcription kit (Promega Corporation, WI, USA). RT-PCR was performed with QuantStudio RT-PCR Instrument (Applied Biosystems, Foster City, CA, USA) using SYBER Green Supermix (Takara, Bio Inc., Otsu, Japan). The primers used are shown in Table 1.

Fecal and serum endotoxin
Endotoxin levels in feces and serum were measured based on the instruction of Mouse endotoxin ELISA Kit (Fudean of Beijing technology CO., LTD., Beijing, China). Standard solution and
Figure 1. Butyrate intervention regulated expression of proinflammatory cytokines. (A) IL-1β; (B) IL-6; (C) MCP1/CCL2; (D) TLR4. NCD, normal chew diet; HFD, high-fat diet; HB, high-fat diet with sodium butyrate. n = 5. Data were analyzed by Mann–Whitney U test. *P < 0.05.

RESULTS

Butyrate intervention suppressed inflammation

Compared with the NCD group, the HFD group exhibited significantly higher mRNA levels of proinflammatory cytokines in liver, including IL-1β, and MCP1/CCL2 (Fig. 1). But butyrate intervention significantly inhibited expressions of IL-1β, IL-6 and MCP1/CCL2 in liver, as well as TLR4 in adipose tissue, compared to the HFD group.

Butyrate intervention modulated β diversity of gut microbiota

Rarefaction analysis indicated that the bacterial diversity was mostly covered under experimental sequencing depth (Fig. 2A). Phylogenetic similarity among different groups was discriminated using β diversity based on principal component analysis (PCA). The first principal component (PC1) and the second principal component (PC2) accounted for 18.45% and 8.41% of the overall variance, respectively (Fig. 2B). The samples collected at 0 weeks were clearly separated from that collected at 8 weeks. And a tendency of the samples in the NCD, HFD and HB groups at 8 weeks to separate into different clusters was observed. Our result indicated that HFD and butyrate intervention changed communities of gut microbiota.

Butyrate intervention modulated gut microbiota composition

A closer insight into the communities of gut microbiota demonstrated that Erysipelotrichaceae and Bacteroidales S24-7 are the dominant families in all the three groups (Fig. 2C). And butyrate intervention significantly increased the abundance of Erysipelotrichaceae family compared with the HFD group. Furthermore, compared with NCD, HFD significantly increased the

Statistical analysis

Mann–Whitney U test was used to determine significance between different groups. SPSS 22.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data was shown as average and standard error. Significance was accepted at P < 0.05.
Figure 2. Butyrate intervention modulated communities of gut microbiota. (A) Rarefaction analysis. (B) Principal component analysis (PCA). (C) The relative abundance of gut microbial families. (D) Correlations analysis between fecal endotoxin and Desulfovibrionaceae abundance. (E) Correlations analysis between serum endotoxin and Desulfovibrionaceae abundance. NCD, normal chew diet; HFD, high-fat diet; HB, high-fat diet with sodium butyrate; 0, before diet intervention; 1, after diet intervention. n = 5.

relative abundance of Ruminococcaceae, Desulfovibrionaceae, Erythrobacteraceae and Bacillaceae families, whereas butyrate intervention reduced the proportion of these four families. Additionally, butyrate intervention significantly decreased the relative abundance of Bacteroidales S24-7, Lachnospiraceae, Bacteroidaceae, Lactobacillaceae, Rikenellaceae, Gemmatimonadaceae, Nitrosporangiacae, Desulfovibrioaceae, Nocardioidaceae, Methylbacteriaceae, Micromonosporaceae, Erythrobacteraceae, Bradyrhizobiaceae, Bacillaceae, Peptococcaceae, Planctomycetaceae, Desulfurellaceae, Paenibacillaceae and Propionibacteriaceae families. Moreover, HFD significantly increased the relative abundance of Ruminiclostridium and Desulfovibrio genera compared with the NCD group, whereas butyrate intervention significantly decreased the relative abundance of Ruminiclostridium, Desulfovibrio, Allobaculum and Bacteroides genera compared with the HFD group (Fig. 3, P < 0.05).

Butyrate enhanced SCFAs and inhibited endotoxin levels

The levels of SCFAs, including acetate, propionate and butyrate, were all reduced by HFD, and the total SCFA concentration was also significantly decreased by HFD (Fig. 4A). However, butyrate intervention significantly increased the levels of propionate, butyrate and the total SCFAs. Moreover, ~2-fold increase of endotoxin levels in feces and serum was observed in the HFD group compared with the NCD group (Fig. 4B). Compared with the NCD group, endotoxin levels in feces and serum after butyrate intervention were reduced by 46.67% and 36.40%, respectively. Specifically, a significantly positive correlation was observed between fecal endotoxin level and Desulfurellaceae abundance (Fig. 2D and E).
Figure 3. Butyrate intervention changed the relative abundance of gut microbial genera. (A) Allobaculum; (B) Ruminiclostridium; (C) Bacteroides; (D) Desulfovibrio. NCD, normal chew diet; HFD, high-fat diet; HB, high-fat diet with sodium butyrate. n = 5. Data were analyzed by Mann–Whitney U test. *P < 0.05.

DISCUSSION

Butyrate intervention selectively changed composition of gut microbiota. In the present study, butyrate intervention decreased potentially pathogenic species, including Desulfovibrionaceae, which was a potential group of endotoxin producers (Zhao et al. 2017). In agreement, our result also demonstrated a significantly positive relation between endotoxin level and Desulfovibrionaceae abundance, which can generate hydrogen sulfide and result in mucosal injury. Moreover, butyrate increased Erysipelotrichaceae proportion; some species of Erysipelotrichaceae are SCFA producers and are also associated with carbohydrate digestion (Van den Abbeele et al. 2018). Zhang et al. (2016) observed that the abundance of Erysipelotrichaceae family has immunogenic potential, and some taxa in this family increased in some diseases caused by inflammation, such as inflammatory bowel disease (Schaubeck et al. 2016) and chronic HIV infection. Previous results indicated that butyrate might benefit hosts by selectively inhibiting some conditionally pathogenic bacteria, such as Desulfovibrionaceae, and promoting some beneficial bacteria, such as butyrate-producing bacteria. The mechanism of butyrate regulating gut microbiota might be because of the reconstructed environment, lower pH, higher levels of butyrate and harmful/beneficial metabolites driven by butyrate supplementation and utilization.

Endotoxin is a constituent of the outer membrane of Gram-negative bacteria, which was linked to high-fat diet-induced inflammation and metabolic disorders (Cani et al. 2008). Our result showed that butyrate intervention decreased endotoxin level in feces and serum, which was consistent with the result reported by Zhou et al. (2017). Furthermore, Schachter et al. (2018) showed that endotoxin secreted by gut microbiota might trigger chronic inflammation in obese individuals. When gut barrier integrity decreased by high-fat diet, endotoxin could transport into circulation via transcellular pathway (Schachter et al. 2017), and it further combined with TLR4 on the surface of immune cells, through MyD88 and TRIF pathway signal transmission, stimulated the expression of a variety of inflammatory factors (Xiao et al. 2014), which was consistent with our results. Taken together, our results suggested that butyrate intervention might suppress inflammation through decreasing endotoxin level.

SCFAs are important links between dietary carbohydrates and host physiology. Lacking of SCFAs might result in some diseases, including type 2 diabetes mellitus (Karlsson et al. 2013). In our study, butyrate intervention increased SCFA levels, including propionate, butyrate and total SCFAs, and this finding was
similar with other intervention studies using dietary fiber (Josue et al. 2018; Zhai et al. 2018), which could be fermented by gut microbiota and give rise to SCFAs (Lin et al. 2012). One of the mechanisms underlying butyrate immunomodulation effects exerted by gut microbiota is through SCFAs (Rescigno 2014). SCFAs have anti-inflammatory ability by stimulating generation of regulatory T cells, as well as by inducing histone H3 acetylation (Smith et al. 2013).

One pathway underlying immune modulation of butyrate is through microbiota-dependent metabolites and components, such as SCFAs and endotoxin, and the other pathway is via reducing expression of proinflammatory cytokines, of which IL-1β, IL-6 and MCP1/CCL2 in liver, as well as TLR4 in adipose tissue were suppressed by butyrate (Fig. 5). Although many studies have shown that butyrate can reduce inflammation through TLR4 pathway (Iraporda et al. 2015; Zhou et al. 2017), significantly lower mRNA level of TLR4 was found only in adipose tissue in our study. Besides suppression the expression of proinflammatory cytokines, the directly anti-inflammatory properties of butyrate have been shown to be mediated by several pathways, such as inducing expression of transforming growth factor-B (TGF-B), inhibiting activities of nitric oxide synthase and metalloproteinas, as well as reducing proliferation and activation of lymphocyte (Bedford and Gong 2018).

**CONCLUSIONS**

Butyrate intervention selectively modulated gut microbiota, such as increasing SCFA-producing bacteria and decreasing endotoxin-secreting bacteria. Butyrate intervention inhibited the expression of proinflammatory cytokines, increased the levels of SCFAs and decreased endotoxin levels, which were positively correlated with Desulfovibrioaceae abundance. In conclusion, butyrate exerted anti-inflammatory effects not only via inhibiting expression of proinflammatory cytokines but also through selectively modulating gut microbiota and microbiota-dependent metabolites and components in high-fat diet-fed mice, such as SCFAs and endotoxin. This work provides underlining information that can be used to explain the effects of prebiotics, and theoretical support for utilization of butyrate in feed additive.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSLE online.

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**Conflict of interest.** None declared.

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