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Original Communication

Fructooligosaccharide Upregulates Colonic Vitamin D Receptors and Modulates Inflammatory Status in High-Fat Diet-Induced Obese Male C57BL/6 Mice

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Abstract

Background: Vitamin D (VD) deficiency is commonly observed in obesity, which may increase morbidity risk. This study explores the effect of fructooligosaccharide (FOS) on VD signaling and inflammatory status in diet-induced obese mice. **Methods**: Therefore, 5-week-old male C57BL/6J mice were randomly assigned (n = 10/group) to groups that received either a (1) standard purified maintenance AIN-93G control diet (CON), (2) CON + 5% FOS (CON-FOS), (3) high-fat diet (HFD) of which 60% kcal was from fat, or (4) HFD + 5% FOS (HFD-FOS) for 10 weeks. **Results**: Mice fed an HFD exhibited reduced serum 25-hydroxycholecalciferol (25D) levels ($p < 0.01$) and a 70% decrease in the expression of colonic *vitamin D receptor* (*Vdr*) mRNA ($p = 0.018$) compared to the mice fed the CON. A 2-fold increase in colonic *Vdr* mRNA expression was observed in the mice fed the HFD-FOS compared to the HFD ($p < 0.01$), although the increased FOS did not alter the serum 25D levels in the HFD group. The mRNA and protein expression of colonic Toll-like receptor 4 (Tlr4) was downregulated in the HFD-FOS group compared to the HFD group, which was negatively correlated to colonic *Vdr* expression (r = –0.747; $p < 0.001$). Additionally, the addition of FOS resulted in a 44% reduction in circulating proinflammatory cytokine Interleukin-6 (IL-6) in the HFD group ($p < 0.01$). The observed upregulation of *β-defensin 1* ($p = 0.017$) and zona-occluden 1 (*Zo-1*) mRNA expression in mice fed FOS and an HFD compared with the HFD group further suggests that FOS supplementation can improve epithelial barrier integrity in HFD-induced obese mice. **Conclusions**: Our data suggest that FOS may be a potential dietary strategy for preventing obesity-induced complications.

Keywords: fructooligosaccharide; vitamin D; obesity; high-fat diet; toll-like receptor 4; colonic inflammation

1. Introduction

To date, approximately 42% of American adults (aged 20 or older) are obese [1], highlighting the need of dietary strategies to prevent obesity and/or obesity-associated complications. Among the obese population, the prevalence of vitamin D (VD) deficiency was 35% greater compared to non-obese individuals [2]. VD is a fat-soluble vitamin that plays a critical role in calcium homeostasis. VD enters the liver where it is converted into 25-hydroxycholecalciferol (25D), also known as calcidiol, by the enzyme 25-hydroxylase. From the liver, 25D is transported to the kidney via VD binding proteins for further hydroxylation by 1-hydroxylase, which is converted into 1,25-dihydroxycholecalciferol (1,25D). 1,25D is a ligand of the VD receptor (VDR) to initiate gene transcriptions for regulation of various physiological functions [3,4]. In recent years, it was suggested that the health benefits of VD could extend beyond bone health. Studies have shown that low level of VD (serum 25D <30 ng/mL) is strongly correlated with incidences of inflammatory bowel disease, cancers, autoimmune diseases, and immune health [5–7]. Specifically, it is estimated that 55% of those diagnosed with ulcerative colitis were classified as obese or overweight, which subsequently increases their risk for hospitalization and results in negative prognosis compared to healthy individuals [8].

Obesity is often characterized by low-grade inflammation, which is usually associated with gut dysbiosis. Studies have shown an increase in Firmicutes and a decrease in Bacteroidetes in an obese intestinal environment [9–11]. In addition, the Western diet and obesity have been associated with a decrease in gut microbiota diversity [12,13] and a high-fat diet (HFD) has been linked to decreased *Bifidobacterium* and increased gram-negative phylum that may lead to the production of lipopolysaccharides (LPS) [14,15]. LPS is a part of the membrane of the gram-negative bacteria and functions as a ligand for Toll-like receptor 4 (TLR4) to enhance pro-inflammatory reaction. Consumption of a HFD or typical Western diet have been linked to increased levels of circulating LPS, likely due to compromised intestinal barrier integrity resulting from epithelial mucosa damage that allows the translocation of endotoxin. This could further promote local and systemic inflammation leading to a condition that is known as metabolic endotoxemia [16–

18]. It is also important to note that gut microbiota can serve as a regulator of antimicrobial peptides productions (AMPs), such as alpha and beta defensins, to regulate the innate immune response within the gastrointestinal tract [19–22]. Probiotics and microbial metabolites, such as short chain fatty acids, have been shown to promote the expressions of tight junction proteins and AMP productions *in vivo* [23,24], suggesting a vital role of gut microbiota in maintaining the integrity and innate response of the gut epithelium.

Fructooligosaccharide (FOS), a soluble fiber also known as oligofructose, contains approximately 2–60 units of fructose molecules linked by beta (2,1) glycosidic bonds [25,26], which allows FOS to be fermented by gut bacteria and resist enzymatic digestion. FOS can be found in multiple plant sources such as onions, wheat, and chicory and has been shown to exert bifidogenic potential [27–29]. Administration of FOS has been linked to improve metabolic health, such as improvement of glucose and lipid metabolism [30–32]. Our previous work demonstrated that intake of resistant starch, a prebiotic fiber, prevented renal VD loss and morbidities in diabetic rats [33,34]. These findings suggest that restoration of VD balance could be beneficial in preventing secondary complications resulting from metabolic diseases. Because resistant starch is a fermentable fiber, the gut microbiota is thought to constitute one of the underlying mechanisms. In support of this concept, we have further demonstrated that supplementation of FOS and VD upregulated the colonic VDR expression in lean mice compared to those fed on VD alone [35,36]. Furthermore, the regimen suppressed the abundance of a pathobiont, *Romboutsia ilealis*, altered the beta diversity of gut microbiome, and modulated the expressions of intestinal defensins, a class of AMPs [36]. Taken together, this may suggest that FOS could potentially activate colonic vitamin D signaling to regulate innate immune response, possibly via modulation of the gut microbiota.

Though we have demonstrated that the combination of FOS and VD can regulate intestinal permeability and innate response through intestinal VDR activations [36], the mechanism by which FOS and/or its fermented by-products can regulate VD homeostasis under an obesogenic environment remains unclear. Because activation of vitamin D signaling has shown to modulate the gut microbial profile and protect against gastrointestinal diseases [37,38], it is speculated that FOS can serve as an effective dietary intervention for prevention obesity-associated inflammation by modulating VD signaling pathway. This study aims to investigate the effect of FOS on colonic VD signaling in relation to their metabolic changes and inflammatory status utilizing a HFD-induced animal model. We expect that the information generated from this study can further elucidate the role of gut microbiota on bioavailability of essential nutrients and serve as a novel strategy to improve quality of life among populations who are susceptible to VD deficiency.

2. Materials and Methods

2.1 Animal Study

In this experiment, five-week-old male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and all mice were individually caged and acclimated for one week. Diets were purchased from Research Diets, Inc (New Brunswick, NJ, USA) and FOS was supplied by Beneo (Orafti-P95, Mannheim, Germany), composed of ~95% of oligofructose derived from chicory root, to Research Diets for customization. All diets were irradiated. At 6-week of age, mice were randomized into 4 groups (n = 10/group) to receive either a control AIN-93G diet (CON), high-fat diet of which 60% kcal was from fat (HFD; D12492), CON + 5% w/w FOS (CON + FOS), and HFD + 5% w/w FOS (HFD + FOS) for 10 weeks. Both the CON and HFD diets contain a base concentration of 1000 IU/kg of VD. The selected FOS dosage has been shown to upregulate the expression of colonic VDR in our previous study [35,36], alter metabolic phenotypes, as well as induce the abundance of *Bifidobacterium* [39,40]. All mice had unlimited access to water and their assigned diet throughout the experimental period. Body weight and food intake were monitored and recorded weekly. By the end of the 10th week of intervention, mice were subjected to overnight fasting and anesthesized with 3% isoflurane via inhalation. Blood was collected via cardiac puncture, followed by euthanasia by cervical dislocation. Colon and ileum sections were collected, and mucosa was scraped for subsequent analyses. All samples were stored at –80 °C until analysis.

2.2 Analyses of Serum 25D and Serum IL-6

The serum was obtained via centrifugation of whole blood at 1200 ×g for 15 min. Undiluted serum was subjected to 25D analysis via a commercial ELISA kit (#80987, Crystal Chem, Elk Grove Village, IL, USA) according to manufacturer's instructions. Serum concentrations of IL-6 was determined using commercially available ELISA kits (KMC0061, Invitrogen, Waltham, MA, USA). All serum samples used in these ELISA assays were undiluted and samples were assayed in duplicate. The optical density was read with a microplate reader at 450 nm (BioTek Cytation5, Agilent Technologies Inc, Santa Clara, CA, USA).

2.3 Analysis of Serum LPS

Mouse serum was prepared from whole blood via centrifugation at 1200 ×g for 15 min. Serum was diluted by 20-fold and lipopolysaccharide endotoxin level was measured in mouse serum using a commercially available Limulus Amebocyte Lysate Chromogenic Endotoxin Quantification Kit (A39552, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. The optical density was obtained at 405 nm with a microplate reader (BioTek Cytation5, Agilent Technologies Inc, Santa Clara, CA, USA).

2.4 Real-Time PCR

Colonic and ileal mucosa were scraped and homogenized prior to total RNA extraction using TRIzol® reagent (#15-596-026, Thermo Fisher Scientific, Waltham, MA, USA). RNA concentrations were determined using a Nanodrop Lite (Thermo Fisher Scientific, Waltham, MA, USA) and cDNA was synthesized using the Superscript® IV First-Strand Synthesis System (#18-090-050, Invitrogen, Waltham, MA, USA). Amplification of target transcripts was obtained via Power up SYBR® Green Master Mix and Quant Studio 3® real-time PCR system (A25776, Thermo Fisher Scientific, Waltham, MA, USA). Primers for all target genes used in this study are listed in **Supplementary Table 1**. The expression of each target gene was normalized against glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and relative expression compared with the CON group was calculated.

2.5 Western Blot

Colonic mucosa was homogenized in 1X RIPA buffer (PI89900, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease and phosphatase inhibitors (PIA32961, Thermo Fisher Scientific, Waltham, MA, USA) and total protein was extracted. Protein quantification was obtained using the BCA assay (#23227, Thermo Fisher Scientific, Waltham, MA, USA). A total of 50 µg of protein sample was loaded on a 4–12% gradient sodium dodecyl sulfate–polyacrylamide gel and transferred onto a nitrocellulose membrane (PI88014, Invitrogen, Waltham, MA, USA). Membranes were probed with primary TLR4 (NB100-56580SS, Novus Biologicals, Centennial, CO, USA) and GAPDH (SC32233, Santa Cruz Biotechnology, Dallas, TX, USA) antibodies, followed by incubation with anti-rabbit (AP187MI, MilliporeSigma, Burlington, MA, USA) or anti-mouse (#45-000-692 Cytiva, Marlborough, MA, USA) secondary antibodies. All antibodies were diluted to 1:1000, unless otherwise specified. Proteins were visualized with enhanced chemiluminescence reagent (PI34094, Thermo Fisher Scientific, Waltham, MA, USA) using a Gel-Doc Image Analysis System (Fotodyne Incorporated, Hartland, WI, USA). Densitometry of protein bands were quantified using ImageJ software (version 1.54p, National Institutes of Health, Bethesda, MD, USA) and data are expressed as ratio relative to CON.

2.6 Statistical Analysis

Sigma Plot v. 14.5 (Inpixon, Palo Alto, CA, USA) was used for all statistical analyses. All endpoints were analyzed using two-way analysis of variance followed by Tukey's Honestly Significant Difference (HSD) post hoc test, which adjusts for multiple comparisons and controls the family-wise error rate. Data for colonic TLR4 protein expression were not normally distributed; hence, data were log-transformed for analysis to achieve normality (p = 0.765). Statistical analyses of mRNA expression data were determined with cycle threshold and expressed in relative to CON as indicated. Correlation between colonic *Vdr* and colonic *Tlr4* mRNA expressions were determined by Pearson's correlation coefficient. Statistical significance was indicated at $p < 0.05$. All results are expressed as mean $\pm$ standard error of mean (SEM), unless otherwise indicated. Western blot data are representative of 2 independent experiments.

3. Results

3.1 FOS Upregulates Colonic Vdr in HFD-Fed Mice Despite no Changes in Vitamin D Status

HFD mice exhibited lower VD status, as measured by serum 25D, compared to CON mice ($p < 0.001$; Fig. 1A). Interestingly, FOS suppressed body weight gain in HFD mice by 10% compared to those fed the HFD diet alone, with no impact on the CON mice (data not shown); yet, FOS did not affect serum 25D levels in either CON or HFD mice (Fig. 1A). Similar to serum 25D levels, a 70% lower colonic *Vdr* mRNA expression was observed in HFD mice compared to CON mice ($p = 0.008$). However, FOS upregulated the mRNA expression of colonic *Vdr* in HFD mice by 2-fold compared to mice on HFD diet alone ($p < 0.01$) (Fig. 1B). In the ileum, *Vdr* mRNA expression was 2.5-fold higher in the HFD mice compared to the CON mice (p = 0.012), though no difference was detected in FOS-treated HFD mice compared to HFD mice (Fig. 1C).

3.2 FOS Attenuates Circulating IL-6 and Modulates the mRNA Expressions of Colonic Pro-Inflammatory Cytokines in HFD-Fed Mice

We further measured the circulating levels of pro-inflammatory cytokines, IL-6 and IL-1β. However, serum IL-1β levels were below the detection limit and hence the result was not obtained. With regards to serum IL-6 (Fig. 2), the circulating level in HFD-fed mice was 2-fold greater than in CON mice ($p < 0.001$), and the administration of FOS suppressed the elevation of circulating IL-6 by 44% in HFD mice ($p < 0.01$), which did not differ from CON mice (Fig. 2). No difference was detected in CON-FOS mice compared to CON mice (Fig. 2).

To further evaluate the local inflammation within the colon, mRNA expressions of selective pro-inflammatory cytokines were measured. In contrast to circulating IL-6 concentration, colonic mRNA expression of *Il6* in HFD mice was lower compared to CON mice (p = 0.066), and that HFD-FOS mice was 4-fold greater compared to HFD-fed mice, though it was not statistically different (p = 0.079; Fig. 3A). A similar trend was observed with colonic *Il1*β, where HFD downregulated the mRNA expression by 5-fold (p = 0.011), and HFD-FOS upregulated the expression of colonic *Il1*β compared to HFD mice (p = 0.015; Fig. 3B). On the other hand, colonic *Tnf*α among HFD mice exerted 2.5-fold greater in mRNA expression compared to CON mice (p = 0.013), and that treatment with FOS downreg-

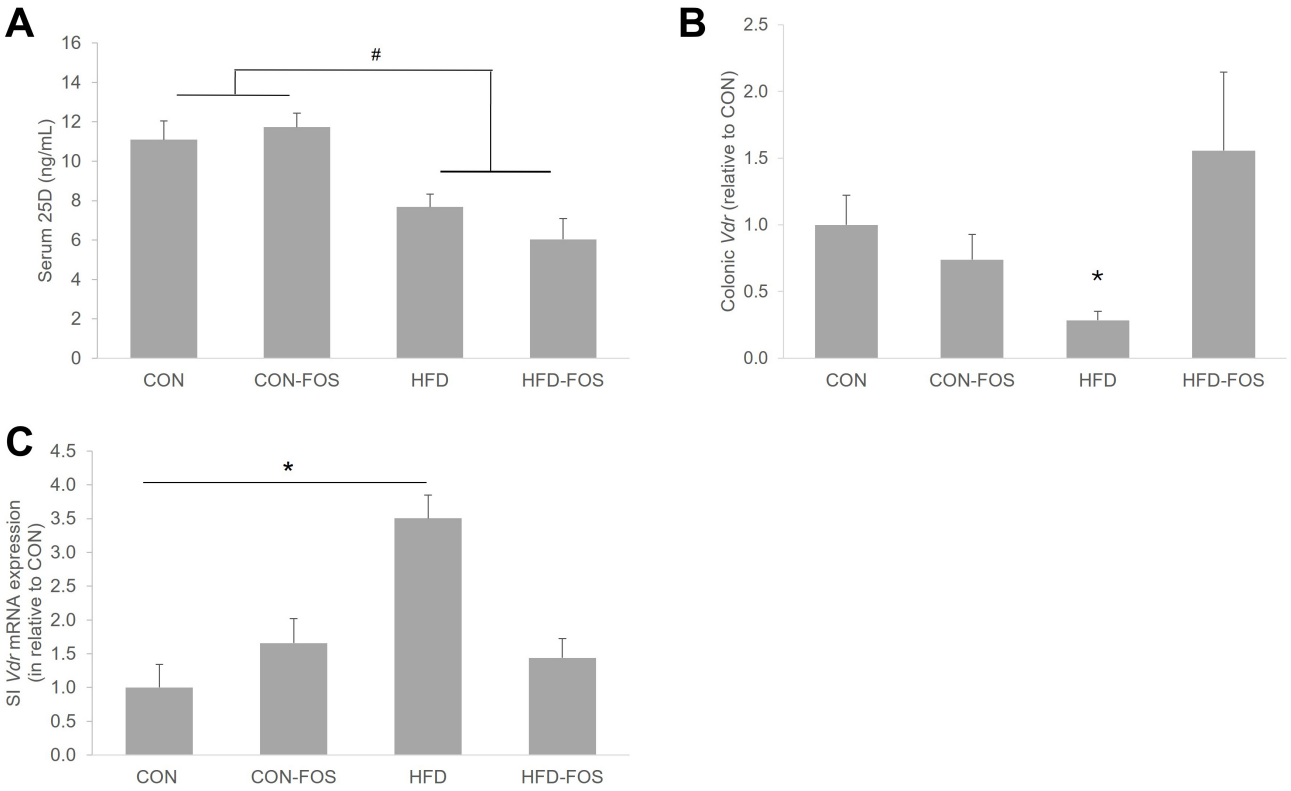

Fig. 1. Circulating 25D in the HFD mice was not impacted by FOS intervention (A), though differential effects of FOS were observed with *Vdr* mRNA expressions in colon (B) and ileum (C) in the HFD mice. Statistical differences between dietary interventions are expressed as $*p < 0.05$. Statistical differences between diet (CON vs. HFD) are expressed as $\#p < 0.05$. Data are expressed as mean ± SEM (n = 5–10/group). CON, mice on a control AIN-93G diet; CON-FOS, mice on CON diet supplemented with 5% FOS; HFD, mice on a high-fat diet; HFD-FOS, mice on a HFD diet supplemented with 5% FOS; FOS, fructooligosaccharides; *Vdr*, vitamin D receptor; 25D, 25-hydroxycholecalciferol; SI, small intestine.

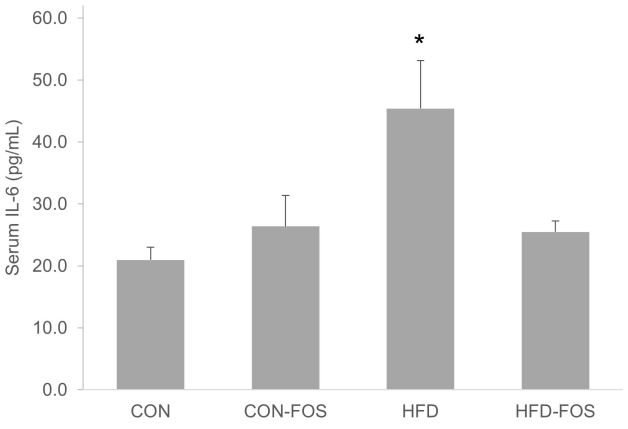

Fig. 2. FOS suppressed the elevation of circulating IL-6 in HFD mice. Statistical differences between dietary interventions are expressed as $*p < 0.05$. Data are expressed as mean ± SEM (n = 7, 8/group). CON, mice on a control AIN-93G diet; CON-FOS, mice on CON diet supplemented with 5% FOS; HFD, mice on a high-fat diet; HFD-FOS, mice on a HFD diet supplemented with 5% FOS; FOS, fructooligosaccharides.

ulated the mRNA expression of colonic *Tnfα* in HFD mice ($p = 0.03$) but not CON mice (Fig. 3C).

3.3 FOS Suppresses the Elevation of Circulating Endotoxin and Colonic TLR4 Expressions Induced by High-Fat Diet

Compared to the CON mice, the endotoxin level, as indicated by circulating LPS concentration, in the HFD mice was 33% higher ($p < 0.01$). Administration of FOS suppressed the level of serum LPS in the HFD mice ($p < 0.001$) to the level similar to CON mice (Fig. 4A). LPS is a common ligand to TLR4. Similar to the serum LPS levels, an increasing trend of mRNA ($p = 0.07$) and protein ($p = 0.01$) expressions of colonic *Tlr4*, was observed in HFD mice, respectively, and that a suppression of colonic *Tlr4* mRNA ($p = 0.011$) and protein ($p = 0.018$) expressions in FOS-treated HFD mice, suggesting a potential attenuation of TLR4 receptor signaling by FOS (Fig. 4B–D). The role of VD in immune regulation is well established. Here, we further demonstrated a negative correlation between colonic *Vdr* and *Tlr4* mRNA expressions ($r = –0.74$; $p < 0.01$), which may indicate a regulation of colonic inflammation upon VDR activation (Fig. 5).

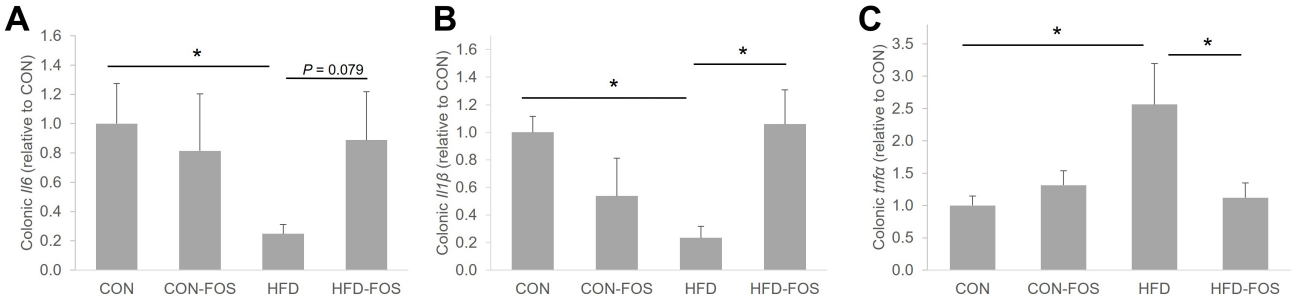

Fig. 3. Colonic *Il6* (A) and *Il1β* (B) mRNA expressions was upregulated and colonic *Tnfα* mRNA expression (C) was downregulated in FOS-treated HFD mice. Data are expressed as mean ± SEM (n = 4–6/group). Statistical differences between dietary interventions are expressed as $*p < 0.05$. CON, mice on a control AIN-93G diet; CON-FOS, mice on CON diet supplemented with 5% FOS; HFD, mice on a high-fat diet; HFD-FOS, mice on a HFD diet supplemented with 5% FOS; FOS, fructooligosaccharides.

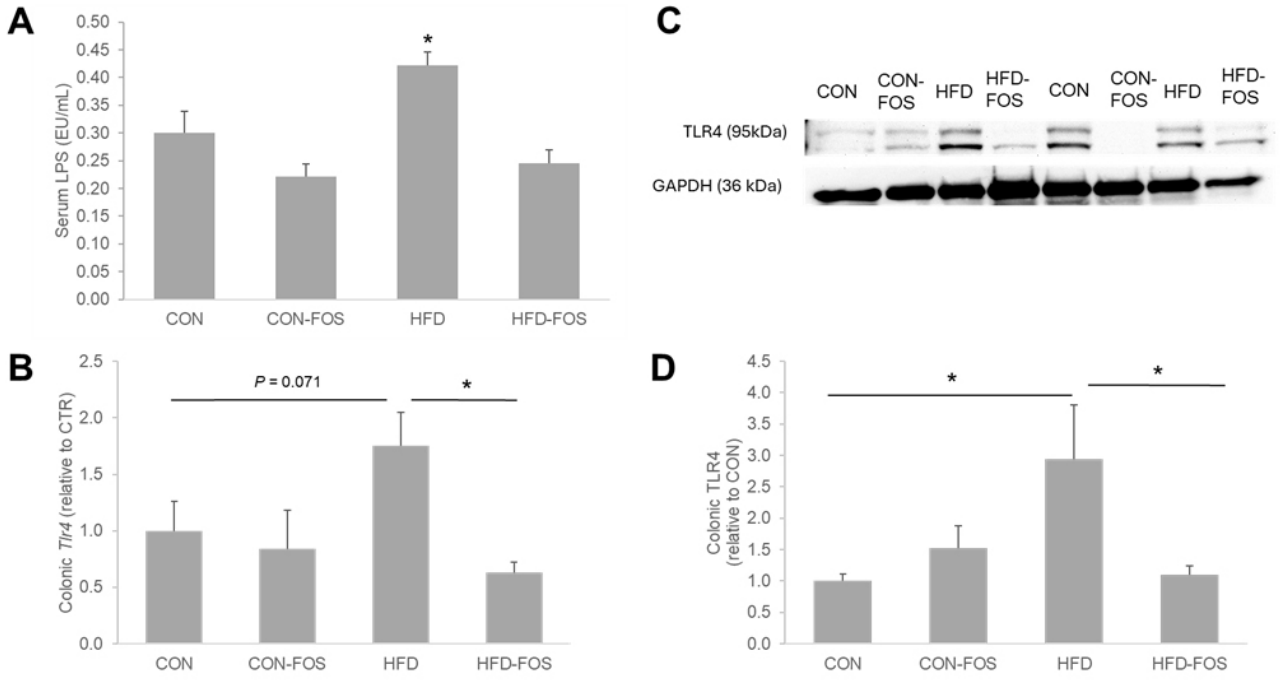

Fig. 4. FOS attenuated the elevation of circulation LPS in HFD mice (A), along with a suppression of TLR4 mRNA (B) and protein (C,D) expressions. (C) Representative Western blot analysis of TLR4. Statistical differences between dietary interventions are expressed as $*p < 0.05$. Data are expressed as mean ± SEM (n = 5–10/group). CON, mice on a control AIN-93G diet; CON-FOS, mice on CON diet supplemented with 5% FOS; HFD, mice on a high-fat diet; HFD-FOS, mice on a HFD diet supplemented with 5% FOS; FOS, fructooligosaccharides; LPS, lipopolysaccharides; Tlr4, toll-like receptor 4.

3.4 FOS Regulates Intestinal Barrier Integrity and Differentially Affects the mRNA Expressions of Anti-Microbial Peptides in HFD-Fed Mice

In our previous study [36], it was demonstrated that treatment with VD in combination with FOS downregulated selected defensins in the ileum of mice fed a control diet suggesting a potential role of FOS in regulating the secretion of these antimicrobial peptides. Here, utilizing the HFD-induced obese model, we demonstrated a diet effect (CON vs. HFD) in both mRNA expressions of *Dfa1* ($p = 0.01$) and *Dfa5* ($p = 0.02$). However, FOS did not affect *Dfa1* nor *DFa5* mRNA expressions in the ileum (Fig. 6A,B), though a trend of *Dfb1* upregulation by almost 10-fold was observed in the FOS-treated HFD mice compared to the HFD mice ($p = 0.017$; Fig. 6C).

VDR has been shown to regulate the secretions of defensins. Our current study confirmed a positive correlation between the mRNA expressions of *Vdr* and *Dfa1* (r = 0.48; $p = 0.03$) and *Dfa5* (r = 0.47; $p = 0.03$) in the ileum, respectively (Table 1). However, no correlation was detected between ileal *Vdr* and *Dfb1* mRNA expressions (Table 1).

The integrity of the gut barrier was indicated by the mRNA expressions of Zona-occluden-1 (*Zo1*) and *occludin* (*Ocln*) in the colon. As expected, HFD downregulated the

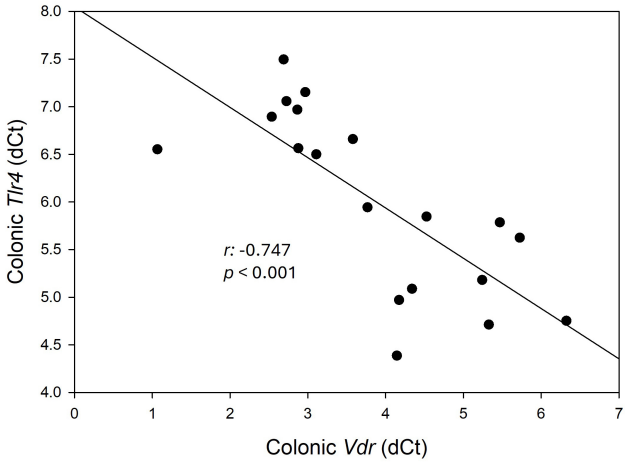

Fig. 5. Colonic *Vdr* mRNA expression is inversely correlation with colonic *Tlr4* mRNA expression. Correlational analysis was conducted using Pearson's correlation. Each dot represents individual data. Data are expressed as mean ± SEM (n = 19). *Vdr*, vitamin D receptor; *Tlr4*, toll-like receptor 4.

Table 1. mRNA expressions of alpha defensins are positively correlated with *Vdr* in the ileum. Correlational analysis was conducted using Pearson's correlation. Each dot represents individual data. Data are expressed as mean ± standard error (n = 20, 21).

Target genes	Pearson's correlation coefficient (r) with ileal *Vdr*	*p*-value
Dfa1	0.487	0.02
Dfa5	0.477	0.03
Dfb1	–0.13	0.57

mRNA expression of colonic *Zo1* by 80% compared to mice on a CON diet ($p < 0.01$; Fig. 7A), and that FOS normalized the colonic *Zo1* mRNA expression in the HFD mice ($p < 0.01$; Fig. 7A). In contrast, an increasing trend was observed with the mRNA expressions of colonic *Ocln* in HFD mice compared to CON mice ($p = 0.057$) and that it was downregulated by FOS in the HFD mice by 50% compared to HFD mice ($p = 0.04$; Fig. 7B).

4. Discussion

Our current study suggests a potential role of FOS in suppressing LPS-induced TLR4 activation in HFD-induced obese mice, likely through a VDR-dependent pathway. Though it is expected to observe low vitamin D status in obesity due to it being sequestered within the adipose tissues [2], our results indicated that 5% FOS was not effective in restoring vitamin D levels within the HFD mice, regardless of body weight changes. However, the FOS administration upregulated colonic *Vdr* among the HFD mice. This is in alignment with our previous study, in which we demonstrated that FOS, in combination with vitamin D supplementation, enhanced the mRNA expression of colonic *Vdr*, though serum 25D was unaltered [35]. Because food intake did not differ among groups, this may suggest that the effect of FOS or gut microbial metabolites derived from FOS fermentation could be targeting the downstream pathway of vitamin D metabolism, possibly the conversion of 25D to 1,25D locally in the colon. Unlike in the colon, we observed a down-regulation of ileal *Vdr* in the FOS-treated HFD mice. This coincides with our previous studies that mRNA expressions of *Vdr* in the colon and ileum are independently expressed despite the status of vitamin D [35,36]. Because FOS fermentation mainly occurs in the colon, this led us to speculate that the differential expressions of *Vdr* along the GI tract could be relevant to bacterial activity and concentrations of microbial metabolites, such as short chain fatty acids, which have been shown to directly regulate VDR activity *in vivo* [41]. A recent study further demonstrated that *Carnobacterium maltaromaticum* can induce the production of intestinal vitamin D to activate VDR for prevention of colorectal cancer [42]. This may partially explain the changes of colonic VDR independent of circulating 25D level and body weight changes in our study. Characterization on relevant vitamin D markers, such as 1,25D concentrations and 1-α hydroxylase may provide further insight to these discrepancies.

It is well characterized in obesity models that elevation of LPS, or metabolic endotoxemia, leads to the activation of TLR4, which could subsequently promote the secretion of pro-inflammatory cytokines [14,15,43]. While our results are in line with the literature, we further demonstrate that the inhibition of colonic TLR4 could be attributed to VDR activation due to the strong inverse correlation between colonic *Tlr4* and *Vdr* mRNA expressions. It is well known that TLR4, when bound to LPS, recruits either TRIF or MYD88 adaptor proteins that ultimately leads to nuclear factor kappa-B (NF-kB) translocation and production of cytokines [44]. The relationship between TLRs and the VDR remains understudied. TLR interplay with VD is primarily understood in the context of immunology in which a co-receptor of TLR4, CD14, is known to be regulated by 1,25D [45]. In addition, *ex vivo* treatment of 1,25D to healthy human monocytes downregulated both mRNA and protein levels of TLR4 and TLR2 [46]. Taken together, these studies may partially explain the inverse relationship we observed in the present study between the mRNA expressions of *Vdr* and *Tlr4*. Another key aspect of TLR-VDR interactions may be their shared modulation of NF-kB transcription. Typically, NF-kB is downstream to TLRs signaling pathway, while VDR modulates NF-kB post- and pre-transcriptionally [47]. VDR can inhibit LPS or TNFα stimulated NF-kB through the binding of IκB kinase. This further limit the phosphorylation of IκB and hence be degraded, leaving NF-kB unable to translocate to the nucleus [47]. Additionally, it has been posed by Wu *et al.* [48], that NF-kB subunit p65 can form a complex with VDR in

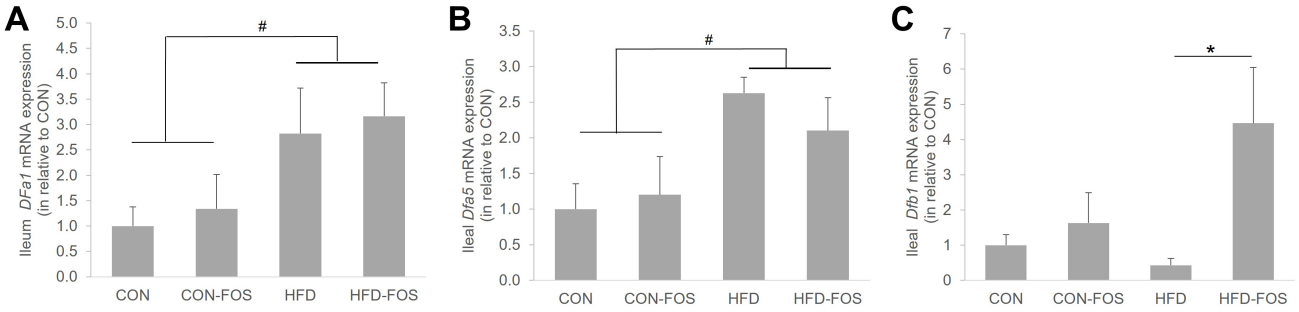

Fig. 6. FOS did not affect the mRNA expressions of ileal *Dfa1* (A) and *Dfa5* (B) but upregulated ileal *Dfb1* mRNA expression (C) in HFD mice. Data are expressed as mean ± SEM (n = 8–10/group). Statistical differences between dietary interventions are expressed as $*p < 0.05$. Statistical differences between diet (CON vs. HFD) are expressed as $\#p < 0.05$. CON, mice on a control AIN-93G diet; CON-FOS, mice on CON diet supplemented with 5% FOS; HFD, mice on a high-fat diet; HFD-FOS, mice on a HFD diet supplemented with 5% FOS; FOS, fructooligosaccharides.

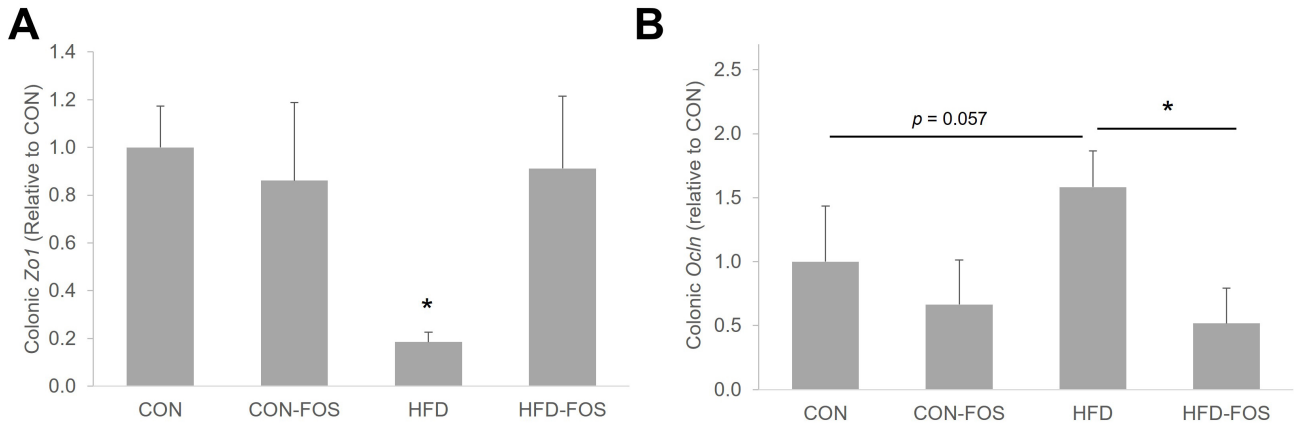

Fig. 7. FOS exerted differential effect on mRNA expressions of colonic *Zo1* (A) and *Ocln* (B). Statistical differences between dietary interventions are expressed as $*p < 0.05$. Data are expressed as mean ± SEM (n = 5, 6/group). CON, mice on a control AIN-93G diet; CON-FOS, mice on CON diet supplemented with 5% FOS; HFD, mice on a high-fat diet; HFD-FOS, mice on a HFD diet supplemented with 5% FOS; FOS, fructooligosaccharides; *Zo1*, Zona-occluden 1; *Ocln*, occludin.

murine colonocytes to inhibit the downstream pathway of NF-kB. Though the exact relationship between VDR and TLR4 is yet to be elucidated, the interactions between these receptors could be mediated by microbial metabolites, such as short chain fatty acids, upon FOS fermentation, which may be crucial in mediating inflammatory responses, especially during the progression of obesity, and warrant further investigations.

Chronic inflammation is often characterized by an increased level of pro-inflammatory cytokines, along with a decreased level of anti-inflammatory cytokines, which is commonly observed during the progression of obesity. At the circulating level, though we were unable to detect IL-1β, we were able to measure the level of IL-6, in which higher level of circulating IL-6 was found in the HFD mice, and that FOS suppressed the elevation IL-6 by almost 2-fold, indicating that FOS may impact the production of pro-inflammatory cytokines in this model. However, at the transcriptional levels within the colon, the expressions of these pro-inflammatory cytokines contrasted with what we would expect, where we observed a decreased mRNA expressions with colonic *Il1*β and *Il6* in the HFD mice, with *Tnf*α having a reverse trend to the other measured cytokines. We speculated that this phenomenon could be due to the compensatory mechanism resulting from overexpression of *Il6*, as evident by increased level of circulating IL-6 concentration, leading to decreased transcriptional activity to prevent overstimulation of the immune system. This is also supported by our previous study in which we have observed an increased levels of pro-inflammatory cytokines accompanied by a decreased mRNA expression of the respective cytokine in a high-fat high-sucrose-fed rat model [49]. Additionally, it has been shown that stress hormones (i.e., cortisol) and anti-inflammatory cytokines may contribute to the negative feedback regulation of pro-inflammatory cytokines [50–52], though further investigation will be required to address such speculation. Because IL-1β was not detected in the serum due to assay limitation, based on the mRNA expression trend shared between *Il1*β and *Il6*, we expect that the regulation of *Il1*β would be similar

to *Il6*. In contrast to *Il1β* and *Il6*, the expression of *Tnfα* in the colon remains elevated in the HFD mice compared to CON mice, which was attenuated by FOS intervention. This may suggest a potential role of Tnfα in actively recruiting other chemokines and cytokines induced by the obesogenic stimulation as Tnfα has been reported to enhance the production of other cytokines, such as IL-6 [53,54]. The regulation of pro-inflammatory cytokines is rather complex and usually depends on specific disease states [55,56]. Our study is limited to the information from these selected pro-inflammatory cytokines, and profiling the chemokines and cytokines family proteins at the transcriptional and post-transcriptional levels under an obesogenic environment will allow us to understand the mechanisms by which FOS can mediate the innate immune response.

Compromised tight junctions can result in bacterial translocation leading to inflammation as previously discussed. Studies have shown that prebiotics or dietary fiber can normalize the intestinal permeability and immune health *in vivo* [57–59]. In the present study, we showed that the protective effects of FOS against obesity-induced low-grade inflammation may be partially mediated through enhanced expressions of beta defensin and tight junction protein, ZO-1. It should be noted that the expressions of colonic *Zo1* and *Ocln* in the HFD mice were differentially impacted by FOS, leading us to speculate that the effect of FOS was specific to ZO-1. This is likely due to the short duration of our study in which the initial destabilization of tight junctions may be primarily involved with the cytoplasmic scaffolding proteins than transmembrane proteins like Ocln. Hence, the observed differences may reflect selective remodeling of tight junction components by FOS rather than restoration of disrupted tight junction.

The role of defensins in promoting epithelial barrier damage in addition to their antibacterial activities has been extensively reviewed and researched [19,20,60]. Compromised expressions of alpha and beta defensins have been reported in patients with Crohn's disease, which further elevates colonic inflammation among these patients [61,62]. Gut microbial metabolites, such as short chain fatty acids, can upregulate the expressions of alpha and beta defensins in GI tract [21,23,63] and hence may promote intestinal barrier integrity. However, in our current study, specifically in the HFD mice, alpha defensins were not affected by FOS intervention, yet the expression of beta defensin was 4-fold greater in FOS-treated HFD mice. The secretions of these defensins can be differentially regulated. For example, specific live microbiota, such as *Enterococcus faecium*, has been shown to promote beta defensin productions [64], while VDR has also been shown to regulate the synthesis of alpha defensin 5 [60]. However, utilizing a HFD-induced obese model in this present study, though we confirmed the role of ileal VDR in regulating the secretions of alpha defensins as reported previously [36], the mRNA expression of *Dfb1* remained unchanged by FOS. While it is beyond our scope of investigation, it is possible that specific gut microbiota resulting from FOS fermentation may cause the enhanced production of *Dfb1*, a pathway that could be independent from VDR activation under obesogenic conditions. However, the impact of the gut microbiota and AMP secretions on intestinal permeability and to what extent remain unclear.

This is the first study demonstrating the protective effect of FOS on obesity-associated colonic inflammation and gut health, possibly via activation of VDR. Limitations in this study are the short duration of interventions and exclusion of female mice in this study. Previously, we reported a significant difference with colonic VDR utilizing a healthy model, hence justified the current study timeline. However, because obesity is a chronic disease, and low-grade inflammation often develops gradually over time, our interpretation of these measured outcomes could be limited. Furthermore, while both male and female mice fed a HFD diet exhibited greater body weight gain compared to those fed a CON diet, FOS attenuated body weight gain only in male mice by ~10%, but not in female (data not shown). Hence, only male mice were included in the study to further our investigations. We further acknowledge the importance of quantifying the proteins levels of critical markers in our study, specifically *Vdr*, to validate the function impact of VDR on obesity-associated inflammation. Due to sample limitations, we were unable to quantify the protein level of VDR, though our previous studies, mRNA expression of *Vdr* has shown strong correlation with our measured outcomes [35,36,49]. Additionally, since FOS is a prebiotic commonly metabolized by gut microbiota, profiling of the gut microbiome and microbial metabolites may provide insight into the mechanisms underlying the protective effect of FOS. However, this is currently beyond our scope of investigations.

5. Conclusions

Collectively, we demonstrated that FOS suppressed HFD-induced TLR4 upregulation in our mouse model, improved intestinal barrier integrity, and potentially stimulated the production of beta defensin. These positive outcomes observed in the colonic microenvironment that is associated with FOS intake could be attributed to the activation of VDR, which lead to subsequent attenuation of TLR4. However, interactions between VDR and TLR4 in regulating colonic inflammation will require further investigation. Due to the immunomodulatory role of VDR, the use of FOS to target VD signaling may serve as a crucial and novel intervention for prevention of metabolic complications in populations who are more susceptible to vitamin D deficiency, such as obesity and Type 2 diabetes. Future research is required to elucidate the interactions between the gut microbiota, vitamin D, and innate immunity following FOS intervention.

Abbreviations

VD, vitamin D; 25D, 25-hydroxycholcalciferol; 1,25D, 1,25-dihydroxycholecalciferol; FOS, fructooligosaccharide; HFD, high-fat diet; CON, control diet-fed; LPS, lipopolysaccharide; TLR4, toll-like receptor 4; IL-6, interleukin-6; IL-1β, interleukin-1β; TNFα, tumor necrosis factor α; ZO-1, zona-occluden 1; Ocln, occludin; AMP, antimicrobial peptides; DFa1, alpha-defensin 1; Dfa5, alpha-defensin 5; Dfb1, beta-defensin 1; NF-kB, nuclear factor kappa-B.

Availability of Data and Materials

All data generated or analyzed during this study are indicated in this article. The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

Conceptualization, GYK; methodology, GYK; formal analysis, KB, EB, SH; investigation, KB, EB, ZN, LL; data curation, GYK; writing—original draft preparation, GYK, KB, SH; writing—review and editing, KB, EB, ZN, LL, SH, GYK; visualization, GYK; supervision, GYK; funding acquisition, GYK. All authors have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animals were handled according to the Guide for the Care and Use of Laboratory Animal (National Research Concils, USA). Animal protocol was approved by the Institute Animal Care and Use Committee at Texas State University, San Marcos, TX 78666, USA with an assigned protocol number 9201.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/IJVNR45457.

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