Impact of oral administration of yeast derived complex dietary-polysaccharide on gut inflammation, microbiota composition, and short-chain fatty acid production

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ABSTRACT

β-Glucans, a group of complex non-digestible polysaccharides, are considered to have beneficial health effects due to their immune modulatory properties and are being widely used as dietary supplements. Prebiotic properties of these polysaccharides in promoting intestinal health are also being recognized. Therefore, we assessed the impact of oral administration of high-pure yeast β-glucan (YBG) on the susceptibility to colitis, gut immune function, composition of gut microbiota and short-chain fatty acid (SCFA) production. We show that prolonged pre-treatment with YBG diminishes the severity of dextran sulfate sodium (DSS)-induced colitis in mice. YBG consumption, compared to untreated controls, results in increased regulatory T cell (Treg) frequencies in the gut mucosa, a shift in the abundance of gut microbiota towards polysaccharide-fermenting bacterial phyla, and higher production of SCFA. Depletion of gut microbiota in YBG-fed mice using broad spectrum antibiotics not only eliminated SCFA production and increased Treg frequencies, but also profoundly enhanced the pro-inflammatory response in the colon and susceptibility to colitis. Overall, our study demonstrates that oral consumption of high-pure BG promotes a healthy gut, immune regulation, minimizes susceptibility to colitis in a microbiota- and SCFA-dependent manner. On the contrary, YBG-consumption when the gut mucosa and microbiota are compromised can reverse this protection and increase the susceptibility to gut inflammation and disease.

INTRODUCTION

Dietary approaches have increasingly been considered for modulating immune function, and preventing autoimmunity and other clinical conditions. Beta-glucans (β-glucans; BGs) are non-digestible complex dietary polysaccharides (CDPs), commonly found in plants, cereals, bacteria, yeast, and fungi. It has been reported that BGs can potentiate the function of immune system and improve the antimicrobial activity of innate immune cells (1, 2). BGs can activate both the innate and adaptive immunity and hence, have been investigated as an effective systemic adjuvant in immunotherapies for cancer and infections (1, 2). Many of the BG-containing preparations have been promoted as daily dietary supplements to enhance immune and metabolic functions (3). In one of our recent reports, we showed that systemic injection of low amounts of highly purified BG [β-1,3-linked D-glucose molecules (β-1,3-D-glucan) with β-1,6-linked side chains], which binds to a C-type lectin, Dectin-1(4, 5), from Baker's yeast (*Saccharomyces cerevisiae*) (YBG) modulates autoimmune progression and prevents type 1 diabetes (T1D) in non-obese diabetic (NOD) mice (6). In another report, we have showed that oral consumption of this BG delays T1D in a spontaneous mouse model by impacting the composition of gut microbiota and systemic immune function (7). While clinical studies using YBG preparations reported beneficial effects (8-11), a clinical trial failed to detect changes in either cytokine production or microbicidal activity of leukocytes upon oral administration of YBG (12). Of note, studies using various BG preparations or BG rich diets have shown that they have the ability to suppress gut inflammation in colitis models (13-17), others have shown that BGs aggravate the colitis severity (18). Furthermore, conflicting results on colitis susceptibility in Dectin-1 deficient mice have been reported by different groups (19, 20). Therefore, additional systematic pre-clinical studies using well defined high pure BGs are needed to fully understand the true BG-dependent impacts of oral consumption on gut immune function and inflammation, and the associated mechanism for designing safe and effective nutriceutical approaches for preventing and ameliorating immune mediated clinical conditions.

In this study, we examined the impact of oral administration of high-pure YBG (1-3,1-6-β-glucan) on gut inflammation using a chemically induced colitis model, and assessed the gut microbiota and short-chain fatty acid (SCFA) associations with YBG consumption-related effects. We show that oral administration of YBG minimizes the severity of dextran sulfate sodium (DSS)-induced colitis in mice. YBG consumption, compared to untreated controls, not only resulted in a shift in the abundances of gut microbiota towards polysaccharide-fermenting bacterial phyla, but also

caused increased production of SCFA. Depletion of gut microbiota in YBG fed mice using broad spectrum antibiotics eliminated the SCFA production, diminished regulatory T cell frequencies and reversed YBG consumption associated protection from colitis. Overall these observations show that oral consumption of high-pure BG promotes gut immune regulation, minimizes susceptibility to colitis in a microbiota- and SCFA-dependent manner, and suggests that well-defined dietary BG preparations may have prebiotic and nutriceutical value in preventing gut inflammation in IBD at-risk subjects with an intact gut microbiota.

Materials and Methods

Mice

C57BL/6 (B6) mice were originally purchased from the Jackson laboratory. Foxp3-GFP-knockin (Foxp3-GFP) mice in B6 background were provided by Dr. Vijay Kuchroo (Harvard Medical School, MA). Breeding colonies of these strains were established and maintained in the pathogen-free facility of Medical University of South Carolina (MUSC). Six-to-ten week old mice were used for experiments. All animal studies were approved by the animal care and use committees at MUSC.

Reagents

Purified YBG (glucan from Baker's yeast, *S. cerevisiae*), ≥98% pure, was purchased from Sigma-Aldrich and the purification method has been described before (21). This agent was tested for specific activity using thioglycolate-activated macrophages as described before (6, 22, 23). PMA, ionomycin, Brefeldin A, and purified and conjugated antibodies were purchased from Sigma-Aldrich, BD Biosciences, eBioscience, Invitrogen, R&D Systems, and Biolegend Laboratories. In some experiments, intestinal epithelial cells were depleted by Percoll gradient centrifugation(24) or using A33 antibody (Santa Cruz Biotechnology, CA) and anti-rabbit IgG-magnetic beads. ELISA and magnetic bead-based suspension array reagents/kits were purchased from R&D Systems, BD Biosciences, Invitrogen, Bio-Rad, Affymetrix-eBioscience and/or Millipore.

Treatments

Age matched female B6 and B6-Foxp3-GFP mice were given YBG suspension in saline (250 μg/mouse/day) for up to 30 days by oral gavage. Control mice were given saline. For depleting gut

microbiota, mice were given a broad-spectrum antibiotic cocktail (ampicillin (1 g/l), vancomycin (0.5 g/l), neomycin (1 g/l), and metronidazole (1 g/l) -containing drinking water during the last 5 days of YBG treatment. In some experiments, antibiotic treatment was continued during colitis induction using DSS.

16S rRNA gene targeted sequencing and bacterial community profiling

Total DNA was prepared from the fecal pellets for bacterial community profiling as detailed in our previous reports (7, 25, 26). DNA in the samples was amplified by PCR using 16S rRNA gene-targeted primer sets to assess the bacterial levels. V3-V4 region of 16S rRNA gene sequencing was performed using Illumina MiSeq platform at MUSC genomic center. The sequencing reads were fed into the Metagenomics application of BaseSpace (Illumina) for performing taxonomic classification of 16S rRNA targeted amplicon reads using an Illumina-curated version of the GreenGenes taxonomic database which provided raw classification output at multiple taxonomic levels. The sequences were also fed into QIIME open reference operational taxonomic units (OUT) picking pipeline (27) using QIIME pre-processing application of BaseSpace. The OTUs were compiled to different taxonomical levels based upon the percentage identity to GreenGenes reference sequences (i.e. >97% identity) and the percentage values of sequences within each sample that map to respective taxonomical levels were calculated. The OTUs were also normalized and used for metagenomes prediction of Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs employing PICRUSt as described before (28-31). The predictions were summarized to multiple levels and functional categories were compared among control and YBG fed groups using the statistical Analysis of Metagenomic Profile Package (STAMP) as described before (29).

Induction of DSS colitis and disease evaluation

To induce colitis, mice were maintained on drinking (tap) water containing 2.5% (w/v) DSS salt (36,000-50,000 MW; MPBio) for 5 consecutive days. After 5 days, mice were switched to regular water until euthanasia on day 10, post DSS treatment initiation. Mice were weighed, and examined for the stool consistency and presence of occult blood every day, and stool consistency and occult blood levels were scored as described before (32). YBG group of mice received the treatment starting day -30 and continued to receive YBG until euthanasia. In some experiments, mice were given antibiotic cocktail starting 5 days before DSS treatment. Mice were euthanized 10 days post-DSS treatment initiation, intestinal tissue were collected, the colon length was measured, and distal

colon tissues were subjected to histochemical staining to determine the severity of inflammation. Distal colon tissues were fixed in 10% formaldehyde, 5-µm paraffin sections were cut, and stained with hematoxylin and eosin (H&E). Stained sections were analyzed using the 0-6 grading criteria described before for inflammatory cell infiltrate, epithelial changes and mucosal architecture (33), and the values were averaged to calculate the overall inflammation score for each mouse. Cells from the intestine and mesenteric lymph node (MLN) were also examined for immune cell phenotype and/or cytokine profiles by FACS and multiplex assay respectively.

Statistical analysis

Mean, SD, and statistical significance (*p-value*) were calculated, graphic visualizations including heatmaps were made using Excel (Microsoft), Prism (GraphPad), Morpheus (Broad Institute), and STAMP (29) applications. Unpaired, two-tailed, *t*-test or Mann-Whitney test was employed, unless specified, for values from *in vitro* and *ex vivo* assays where two groups were compared. All the statistical analyses for microbial sequences were done employing two-tailed *t*-test and the *p*-values were corrected from multiple tests using Benjamini and Hochberg approach in STAMP. Fisher's exact test was employed to compare inflammation severity grades in test and control groups. A *p*-value of ≤ 0.05 was considered significant.

Results

Pretreatment with YBG diminishes susceptibility to DSS induced colitis

B6 mice were given YBG for 40 consecutive days and DSS during the days 30-35 as shown in Fig. 1A, and monitored for colitis associated features including loss of body weight, stool consistency and fecal blood during days 30-40. In addition, colon length and colonic inflammation were determined after euthanasia on day 40. As observed in Fig. 1B, YBG treated mice were relatively less susceptible to weight loss compared to control mice and significant difference in the body weight between control and YBG treated groups was observed starting day 6, post-DSS treatment initiation. YBG treated mice, upon euthanasia, also showed significantly longer large intestine compared to control group of mice (Fig. 1C), suggesting that treatment with this CDP suppresses colitis associated shortening of the colon. Overall histological injury scores in DSS-treated mice that received YBG, compared to control group, was significantly lower. The colon of DSS-treated control mice exhibited more severe inflammatory infiltrates and ulceration (Figure

1D) compared to YBG treated mice. Further, better stool consistency and lesser fecal blood were detected in YBG-fed mice at the time-point in which these features were most severe (Supplemental Fig. 1). Overall, these observations show that pre-treatment with YBG for considerable duration can result in diminished colitis susceptibility. Of note, we also observed that mice that were not pre-treated, but given YBG only during colitis induction, did not show any difference in the severity of colitis associated parameters compared to control mice (Supplemental Fig. 2).

YBG treated and control mice with colitis showed distinct immune characteristics

Since YBG-fed mice showed less severe colitis, T cells from their gut associated MLNs were examined for the associated phenotypic properties in comparison with their counterparts from control mice. Foxp3+ T cell frequencies were profoundly higher in the MLN of YBG-fed mice compared to control mice (Fig. 2A). Importantly, PMA/ionomycin stimulation ex vivo, followed by FACS analyses, showed that IL10+, IL4+ and IL9+ T cell frequencies were significantly higher and IFNγ+, IL17+ and IL22+ T cell frequencies were relatively lower in YBG-fed mice compared to controls. These observations were validated by multiplex cytokine assay that showed similar trend in the levels of these cytokines secreted by MLN cells from YBG fed and control mice, in response to ex vivo activation using anti-CD3 antibody (Fig. 2B). These results suggest that long-term YBG treatment modulates the immune cell phenotype in the gut mucosa.

Long-term YBG treatment results in changes in the fecal microbiota composition

Our recent report showed that YBG treatment can alter the gut microbiota composition in non-obese diabetic mice and suppress the progression of autoimmune type 1 diabetes (7). Since pre-treatment with YBG appears to prime the gut mucosa resulting in reduced colitis susceptibility, we determined if modulated colitis susceptibility of B6 mice that received prolonged pre-treatment with YBG is associated with altered gut microbiota. Pre- and post-treatment fecal samples from treated and untreated mice were examined for changes in the profiles of microbial communities by 16S rRNA gene sequencing. Compilation of OTUs to different taxonomical levels showed that the abundance of major phyla changed significantly in YBG-fed mice, but not in control mice, compared to the pre-treatment time-point (Fig. 3A). Treatment of mice with YBG resulted in an increase in the abundance of Bacteroidetes (albeit not significant statistically), and Verrucomicrobia phyla members and profound decrease in Firmicutes in their fecal samples.

Similar differences in the abundances of Bacteroidetes and Firmicutes were observed when YBG treated mice were compared to untreated controls at post-treatment time-point (Supplemental fig. 3).

Interestingly, examination of OTUs at genus level revealed that the abundance of many major microbial communities are altered significantly after YBG treatment (Fig. 3B). Reduction in the abundance of microbial communities belonging to the Firmicute phylum including *Blautia, Lactobacillus, Clostridium* and *Oscillospira* was observed in YBG-fed mice as compared to the pre-treatment time-point. On the other hand, abundance of *Butyricimonas* and *Sphingobacterium*, both belonging to Bacteroidetes phylum, and several minor communities (not shown) were increased post-YBG treatment. Increase, albeit not significant statistically, in the abundance of Akkermansia (phylum: Verrucomicrobia) and Dysgonomonas (phylum: Bacteroidetes) after YBG treatment was also observed (not shown). Overall, a major shift in favor of Bacteroidetes and Verrucomicrobia members, which includes many polysaccharide-degrading bacterial communities, over Firmicutes occurred after YBG treatment.

For OTU-based prediction of metabolic functions of gut microbes that may be associated with complex dietary polysaccharide degradation and metabolite production, PICRUSt application was employed. Predicted major pathways that are overrepresented post-YBG treatment, which showed significant changes in gut microbial composition compared to pre-treatment time-point, based on 16S rRNA gene identity include carbohydrate metabolism, glycan biosynthesis and metabolism and biosynthesis of secondary metabolites (Fig. 3C). While additional studies are needed to understand if these major metabolic pathways and their constituents contribute to the modulation of gut immune response, overrepresentation of these pathways in gut microbiota of YBG fed mice suggests a selective enrichment of carbohydrate fermenting/consuming- and metabolite-producing bacteria upon YBG consumption.

YBG treatment results in modulation of small and large intestinal immune phenotype

Supplemental fig. 4 shows that the small intestine (ileum), but not large intestine (colon), of mice that received YBG for 30 consecutive days expressed significantly higher levels of pro-inflammatory factor TNFα and immune regulatory enzyme Raldh1A2 compared to that of untreated counterparts. On the other hand, both ileum and colon of YBG treated mice expressed

significantly higher levels of IL10 suggesting that prolonged treatment with YBG could produce effective physiological impacts in vivo, perhaps through different mechanisms in the small and large intestines. These results along with altered composition of gut microbiota and modulated DSS colitis susceptibility upon prolonged treatment with YBG prompted experiments to determine the overall impact of YBG on gut immune phenotype under intact and depleted microbiota. First, mice that received YBG for 30 consecutive days and with intact microbiota were examined for Foxp3 expression and cytokine production by intestinal T cells. As shown in Fig. 4A, significantly higher frequencies of Foxp3+ T cells were detected in mesenteric LNs (MLNs) and small and large intestinal lamina propria (SiLP and LiLP) of YBG treated mice compared to controls. Further, compared to controls, small intestinal cells from YBG treated mice produced significantly higher amounts of IL10 and IL17. On the other hand, colonic immune cells of YBG treated mice produced higher amounts of IL10, but not IL17 or IFNγ. We then depleted gut bacteria in control and YBG recipient mice using broad-spectrum antibiotics (Supplemental fig. 5) to assess the influence of microbiota on YBG consumption-associated increase in Foxp3+ T cells as well as other immune characteristics. Depletion of gut bacteria alone caused substantial reduction in Foxp3+ T cell frequencies in both control and YBG recipient mice (Fig. 4B), relative to their counterparts with intact microbiota (shown in Fig. 4A). Further, SiLP cells, but not LiLP cells, from microbiota-depleted YBG recipient mice showed relatively higher frequencies of Foxp3+ T cells. Interestingly, small intestine of antibiotic-treated YBG-recipients showed higher IL10, IL17 and IFNγ production, compared to microbiota-depleted controls. On the other hand, immune cells from large intestine of YBG-treated microbiota-depleted mice produced significantly higher amounts of IL17 and IFNγ, but not IL10, compared to control counterparts. Overall, these observations show that while YBG treatment may have a direct impact on immune activation and T cell phenotype of small intestine, immune regulatory effects of this treatment on large intestinal immune cells is, at least in part, gut microbiota dependent and depletion of gut microbes results in diminished IL10 production as well as Treg frequencies. Further, gut microbes appear to contribute to the maintenance of overall Treg function in both small and large intestines.

YBG treatment results in increased immune regulatory SCFA production in the gut

Complex non-digestible dietary polysaccharides are thought to be degraded / fermented by colonic bacteria (34-36). Fermentation of non-digestible sugars by colonic bacteria can also result in the

generation of metabolites including SCFA, which are known to play an essential role in the gut integrity and immune health (37, 38). Therefore, we examined fecal SCFA levels in control and YBG treated mice with intact and depleted gut microbiota. As observed in Fig. 5A, fecal SCFA levels, acetic acid, propionic acid and butyric acid particularly, are significantly higher in YBG-treated mice compared to their control counterparts. On the other hand, depletion of gut microbiota not only resulted in the elimination of this YBG-treatment associated effect on SCFA production, but caused profound suppression of overall SCFA levels in both control and YBG treated mice (Fig. 5B). These observations suggest that enhanced immune regulation in the colon and suppressed colitis susceptibility in YBG treated mice could be gut microbiota and increased microbial SCFA production associated.

YBG treatment associated protection from colitis is gut microbe-dependent

Since YBG treatment resulted in significant changes in the composition of gut microbiota and increased SCFA production, the influence of microbiota on YBG treatment-associated protection of mice from DSS-induced colitis was examined. YBG treated and control mice were given broad spectrum antibiotics to deplete gut microbiota, starting 5 days before initiating DSS treatment to induce colitis as shown in Fig. 6A. As observed in Fig. 6B and C, control and YBG treated mice with intact gut microbiota showed weight loss and disease severity comparable to that described in Fig. 1. Importantly, control mice with microbiota depletion showed relatively lesser weight loss and disease severity compared to their control counterparts. On the other hand, YBG-treated microbiota-depleted mice not only showed reversal of YBG treatment associated protection from colitis, but also had higher weight loss and disease severity compared to untreated mice with intact microbiota. Examination of cytokine profiles of immune cells from the colons of these mice revealed significantly higher pro-inflammatory IFNγ and IL17, and comparable anti-inflammatory IL10, production in YBG-treated microbiota depleted mice compared to YBG treated mice with intact gut microbiota (Fig. 6D). Overall, these observations suggest that while YBG treatment associated protection of mice from colitis is microbiota dependent, YBG alone could produce pro-inflammatory effect in the colon under microbiota depletion, perhaps through direct interaction with colonic immune cells under DSS treatment induced inflammation.

Discussion

A large body of literature suggests that certain complex dietary polysaccharides such as BGs of different microbial and plant origin affect the host immune function (39-43). While previous studies using various BG preparations and BG rich diets have shown that they have the ability to suppress gut inflammation in colitis models (13-17), others have shown that BGs aggravate the colitis severity (18). Further, while it has been shown that genetic deletion of a BG interacting receptor, Dectin-1, does not affect the course of murine experimental colitis, another group found Dectin-1 deficiency increased susceptibility to chemical induced colitis (19, 20). Hence, although BGs from different sources can vary structurally and functionally, the true impact of orally administering well defined BGs on gut inflammation and the associated mechanisms are largely unknown. Further, the impact of high pure BGs on gut microbiota function and microbial SCFA production are under investigated. Our previous reports showed that systemic as well as oral administration of low-dose, highly purified YBG prevents type 1 diabetes in NOD mice (6, 7) suggesting that β-1,3/1,6-glucan exposure enhances overall immune regulation under autoimmune susceptibility situations. Recently, we have also shown that consumption of this BG can alter the composition of gut microbiota (7). In the current study, we determined the impact of oral administration of high pure YBG as a dietary supplement on susceptibility to gut inflammation using chemical induced colitis, gut immune function characteristics, fecal microbiota composition and SCFA production. We show that lengthy pre-treatment with YBG before inducing colitis results in significantly lower DSS induced colitis severity, which was associated with enhanced gut immune regulation, altered gut microbiota composition and function favoring polysaccharide metabolism, and increased SCFA production. Our observations suggest that this prebiotic property of YBG can be exploited for minimizing the colitis susceptibility of at-risk subjects with intact gut microbiota.

In spite of some conflicting reports, previous studies have generally suggested that BG containing agents from various sources including polysaccharide preparations from yeast, can ameliorate the colitis susceptibility in rodent models (13-19). Interestingly, our observations from studies that used high pure YBG show that prolonged oral pretreatment/consumption of this agent is essential for suppressing colitis susceptibility and treatment with this agent at disease induction stage alone

has no beneficial effects. This suggests that BG treatment induced protection from colitis may be a gradually acquired indirect effect, perhaps through altering the structure and function of gut microbiota, but not by direct interaction with gut mucosa. In fact, as observed in our recent report which used type 1 diabetes model (7), we found that prolonged oral treatment using low-dose YBG causes changes in the gut microbial composition by reducing the abundance of Firmicutes and increasing the abundance of Bacteroidetes and Verrucomicrobia phyla, which include many polysaccharide fermenting bacteria. Further, predictive functional profiling of fecal microbiota shows overrepresentation of metabolic pathways linked to carbohydrate metabolism, glycan biosynthesis and metabolism, and biosynthesis of secondary metabolites after YBG treatment compared to pre-treatment time-point. More importantly, our novel observations that fecal levels of microbial metabolites such as acetic acid, propionic acid and butyric acid are higher in YBG treated mice compared to controls, and depletion of gut microbiota profoundly diminishes the levels of these SCFAs, substantiate the notion that YBG consumption not only impacts the structure of microbiota, but also its function, and potentially contributes to diminished colitis susceptibility.

Colonic microbial communities can ferment non-digestible dietary polysaccharides and use them as energy sources for their growth (19, 44-46). Therefore, these polysaccharides are being considered as prebiotics with the ability to promote growth of specific beneficial microbial communities. Previous studies have shown that gut colonization by specific commensal microbial communities causes induction and expansion of Foxp3+ Tregs in the intestinal and systemic compartments (47-49). Further, gut bacteria play a critical role in maintaining peripheral tolerance and gut immune homeostasis (50, 51). However, it is not clear if YBG or any other BG shaped gut microbes directly promote Treg induction and/or expansion. Our observations that YBG treatment increases the production of microbial SCFA, which are known to enhance gut integrity and immune regulation (37, 38), suggest that microbiota does contribute to YBG treatment associated increase in Treg frequencies. Further, our observations that depletion of gut bacteria using broad-spectrum antibiotics not only diminishes Foxp3+ T cell frequency compared to mice with intact gut microbiota, but also eliminates the YBG treatment induced increase in the frequency of these cells in the large intestine substantiate this notion. Further, microbial depletion and reduced SCFA production resulted in increased production of pro-inflammatory cytokines IFNγ and IL17 in the distal gut of YBG treated mice indicating diminished immune regulation in them.

Although this study is focused on the impact of YBG treatment on colitis susceptibility and distal gut, we found that, unlike the large intestine, small intestine of both control and bacteria-depleted YBG treated mice have relatively higher frequencies of Tregs compared to their control counterparts. This suggests that YBG-treatment associated increase in Treg abundance in these two gut compartments may involve, at least in part, different mechanisms. It is possible that while YBG interacts directly with small intestinal mucosa, our results indicate that YBG treatment induced enhanced immune regulation in the large intestine could be primarily microbiota and SCFA dependent. Although we previously reported that Dectin-1 dependent interaction of YBG with immune cells could promote a combination of both pro-inflammatory and immune regulatory immune responses (6, 7) under normal circumstances, this direct interaction may produce primarily pathogenic response under depleted microbiota and pro-inflammatory conditions such as a chemical insult using DSS. In fact it has been shown that the degree and type of immune cell response to BGs depends on the cytokine milieu and microenvironment (52). It is possible that YBG is degraded by colonic bacteria and produce immune regulatory SCFA along with minimizing its direct interaction with large intestinal mucosa. On the other hand, microbial depletion could not only diminish YBG degradation and SCFA production, but also expose large intestinal epithelium and immune cells of DSS treated mice to direct interaction with YBG resulting in aggravated inflammation. In fact, this is evident from our observation that YBG treated, microbiota depleted mice presented more severe colitis compared to untreated groups of mice with intact and depleted microbiota.

Overall, our observations show that pre-conditioning of the intestinal microenvironment in terms of altering gut microbiota composition and function, which enhances SCFA production, by treating with YBG diminishes susceptibility to chemical induced colitis under normal circumstances. However, antibiotic treatment which depletes microbiota not only eliminates this protection in YBG treated mice, but also makes them more susceptible to DSS colitis. Hence, we conclude that YBG-like complex polysaccharides have the ability to promote gut and overall immune health as prebiotics, but may produce adverse effects in those who are undergoing antibiotic therapy.

Conflict of Interest statement: Authors do not have any conflict(s) of interest to disclose.

Author contribution: R.B., B.J, and J.S. researched and analyzed data, and R.G. researched and analyzed the data and edited the manuscript; C.V. designed experiments, researched and analyzed data, and wrote/reviewed/edited manuscript.

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Legends for Figures

FIGURE 1: Pre-treatment with YBG results in diminished susceptibility to DSS induced colitis. B6 mice were given saline or YBG suspension (250 μg/mouse/day) for 40 consecutive days by oral gavage. These mice received 2.5% (w/v) DSS in drinking water during days 30-35 and switched to regular water for remaining 5 days as depicted in panel **A**. **B)** Body weight of individual mice was measured starting pre-DSS treatment (day 30) every day. Mean±SD values of changes in total body weights, relative to initial body weight, of a total of 8 mice tested in each group are shown. Statistical significance was calculated by unpaired *t*-test for each time-points. Stool consistency and fecal blood levels of these mice were scored and presented as Supplemental fig. 1 and 2. C**)** These mice were euthanized on day 40 (day 10 post-DSS treatment initiation), colons were harvested and their length was measured. Colons of two representative mice/group (left panel) and Mean±SD values of length of colons from 8 mice/group (right panel) are shown. Statistical significance was calculated by unpaired *t*-test for each time-points. **D)** H&E stained distal colon sections were evaluated for the degree of inflammation and representative images of sections (left panel) and Mean±SD values of inflammation severity scores of 8 mice/group (right panel) are shown. *p*-values by Fishers' exact test comparing number of mice with grade ≤3 and ≥4 inflammation scores.

FIGURE 2: YBG pre-treatment induced protection from colitis is associated with modulation of gut immune response. B6 mice were given saline or YBG suspension, colitis was induced as described for Fig. 1, and MLN cells were harvested for immunological analyses. **A)** Cells were stained for Foxp3 and subjected to FACS analysis. Cells were also activated using PMA/Ionomycin in the presence of Brefeldin A for 4 h and subjected to intracellular staining for cytokines and analyzed by FACS. CD4+ population was gated for determining cell frequencies. Representative FACS plots (left panel) and Mean±SD frequencies of cells positive for indicated specific factors, 6 mice/group tested individually in duplicate, (right panel) are shown. **B)** MLN cells were cultured in the presence of anti-CD3 antibody for 24h and the spent media were subjected to Luminex technology based multiplex assay. Mean±SD values of concentrations of indicated cytokines produced by cells from 6 mice/group tested individually are shown. *p*-values by Mann-Whitney test.

FIGURE 3: Prolonged oral administration of YBG results in changes in the composition of gut microbiome. B6 mice were given saline or YBG suspension (250 µg/mouse/day) for 30 consecutive days by oral gavage as described for Fig. 1 and fresh fecal pellets from individual animals were collected pre- and post-treatment time-points. DNA prepared from the fecal pellet collected before (day 0) and 30 days after YBG treatment (day 30) was subjected to 16S rRNA gene (V3V4) -targeted sequencing using the Illumina MiSeq platform. The OTUs that were compiled to different taxonomical level based upon the percentage identity to reference sequences (i.e. >97% identity) and the percentage values of sequences within each sample that map to specific phylum and genus were calculated. **A**) Relative abundances of 16S rRNA gene sequences in fecal samples of individual mice before and after treatment at phyla level are shown. **B**) Mean relative abundances of sequences representing the major microbial communities (>1 % of total sequences) in YBG treated group at genus level are shown. **D**) Normalized OUT-biom table was used for predicting gene functional categories using PICRUSt application and selected level 2 categories of KEGG pathway are shown. Statistical analyses were done employing two-tailed *t*-test and the *p*-values were corrected from multiple tests using Benjamini and Hochberg approach in STAMP.

FIGURE 4: Prolonged treatment using YBG causes modulation of T cell phenotype. B6 mice were given saline or YBG suspension (250 µg/mouse/day) by oral gavage for 30 consecutive days and maintained on regular drinking water (**A**) or drinking water containing broad-spectrum antibiotic cocktail (**B**) during the last 5 days (days 25-30). **Left panels:** Single cell suspension of MLN or enriched small intestinal and large intestinal immune cells were stained for CD4+ cells with Foxp3 expression, and representative FACS graphs and mean±SD values of 5 mice tested individually are shown. Enriched immune cells from SiLP (middle panels) and LiLP (right panels) were also cultured in the presence of anti-CD3 antibody for 24 h and supernatants were tested for cytokine levels by suspension bead array. *p*-values by Mann-Whitney test.

FIGURE 5: Prolonged treatment using YBG causes modulation of T cell phenotype. B6 mice were given saline or YBG suspension (250 µg/mouse/day) by oral gavage for 30 consecutive days and maintained on regular drinking water (**A**) or drinking water containing broad-spectrum antibiotic cocktail (**B**) during the last 5 days (days 25-30). Fresh fecal pellets collected from individual mice on day 30 were subjected to SCFA analysis. Mean±SD values of SCFA levels in samples from 5 mice tested individually are shown. *p*-value by unpaired *t*-test.

FIGURE 6: Depletion of gut microbiota in YBG treated mice increased DSS induced colitis. B6 mice were given saline or YBG suspension by oral gavage for 40 consecutive days, DSS in drinking water during days 30-40, and evaluated as described for Fig. 1. Cohorts of control and YBG treated mice also received broad spectrum antibiotic cocktail in drinking water during days 25-40 as depicted in panel **A**. **B)** Body weights of individual mice were measured starting pre-DSS treatment (day 30) every day. Mean±SD values of changes in total body weights, relative to initial body weight, of a total of 5 mice tested in each group are shown. Statistical significance was calculated by unpaired *t*-test for each time-points. C**)** H&E stained distal colon sections were evaluated for the degree of inflammation and Mean±SD values of inflammation severity scores of 5mice/group (right panel) are shown. *p*-values by Fisher exact test comparing number of mice with grade ≤3 and ≥4 inflammation scores. D**)** Immune cells enriched from the colon were cultured in the presence of anti-CD3 antibody overnight and the spent media were subjected to multiplex assay. Mean±SD values of concentrations of indicated cytokines produced by cells from 5 mice/group tested individually are shown. *p*-values by unpaired two-tailed *t*-test.

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Fig. 1

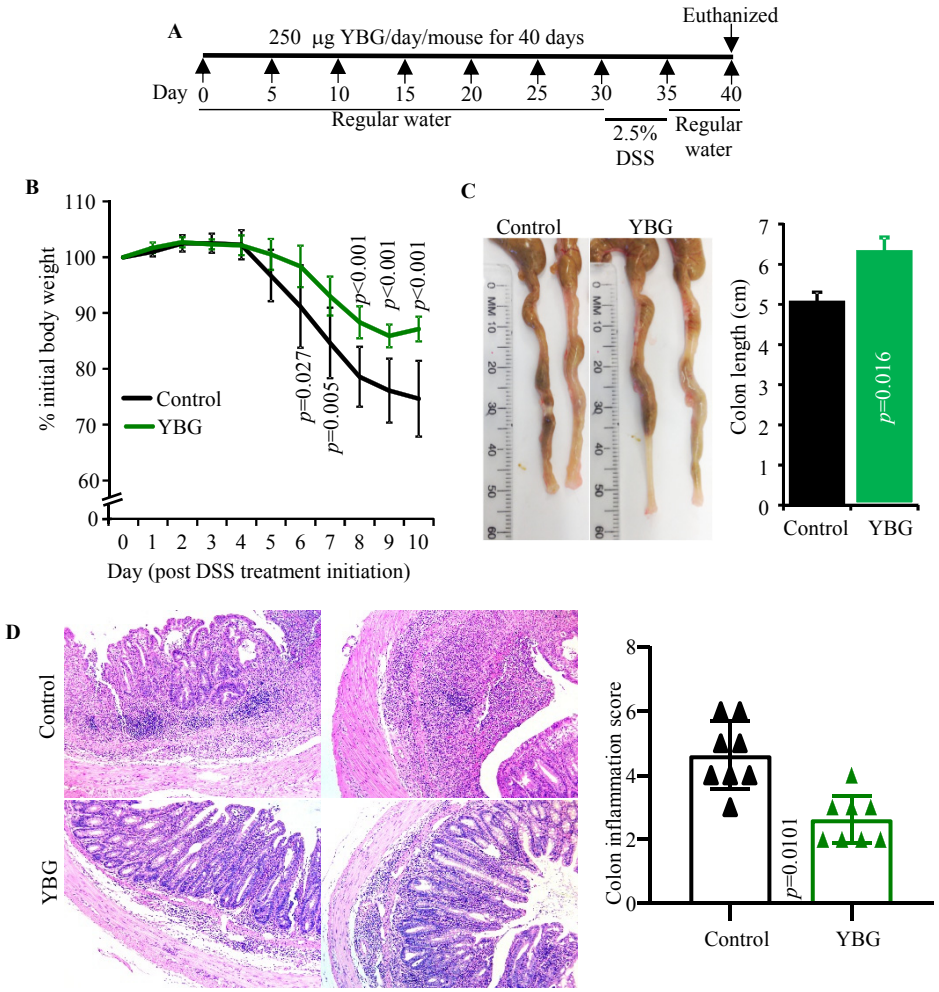

A
250 µg YBG/day/mouse for 40 days
Euthanized
Day 0 5 10 15 20 25 30 35 40
Regular water
2.5% DSS
Regular water
B
% initial body weight
Control
YBG
p=0.027
p=0.005
p<0.001
p<0.001
p<0.001
Day (post DSS treatment initiation)
C
Control
YBG
Colon length (cm)
p=0.016
Control
YBG
D
Control
YBG
Colon inflammation score
p=0.0101
Control
YBG

Fig. 2

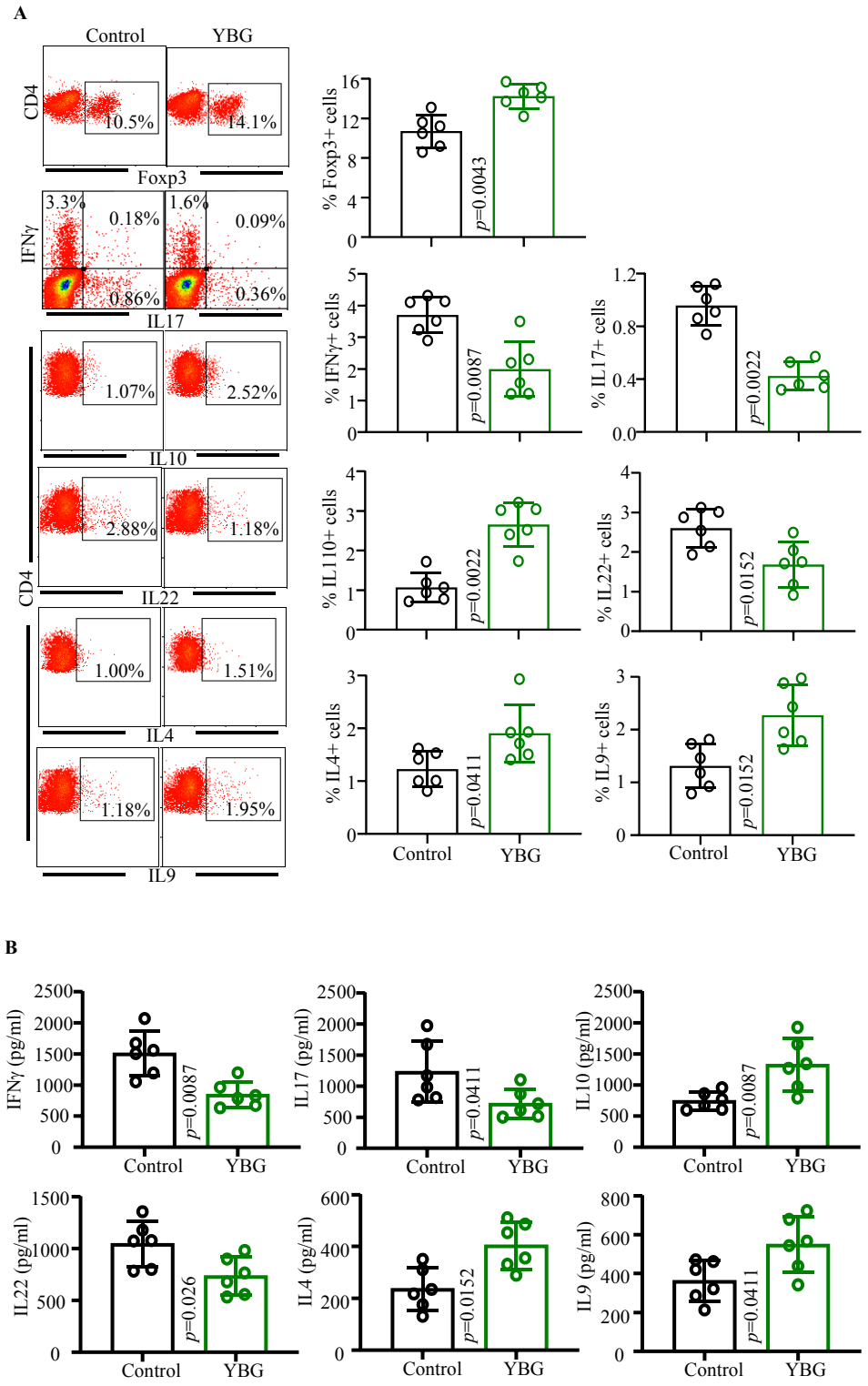

A
Control
YBG
CD4
10.5%
14.1%
Foxp3
IFNγ
3.3%
0.18%
0.86%
1.6%
0.09%
0.36%
IL17
1.07%
2.52%
IL10
2.88%
1.18%
IL22
1.00%
1.51%
IL4
1.18%
1.95%
IL9
% Foxp3+ cells
p=0.0043
% IFNγ+ cells
p=0.0087
% IL17+ cells
p=0.0022
% IL110+ cells
p=0.0022
% IL22+ cells
p=0.0152
% IL4+ cells
p=0.0411
% IL9+ cells
p=0.0152
B
IFNγ (pg/ml)
p=0.0087
IL17 (pg/ml)
p=0.0411
IL10 (pg/ml)
p=0.0087
IL22 (pg/ml)
p=0.026
IL4 (pg/ml)
p=0.0152
IL9 (pg/ml)
p=0.0411

Fig. 3

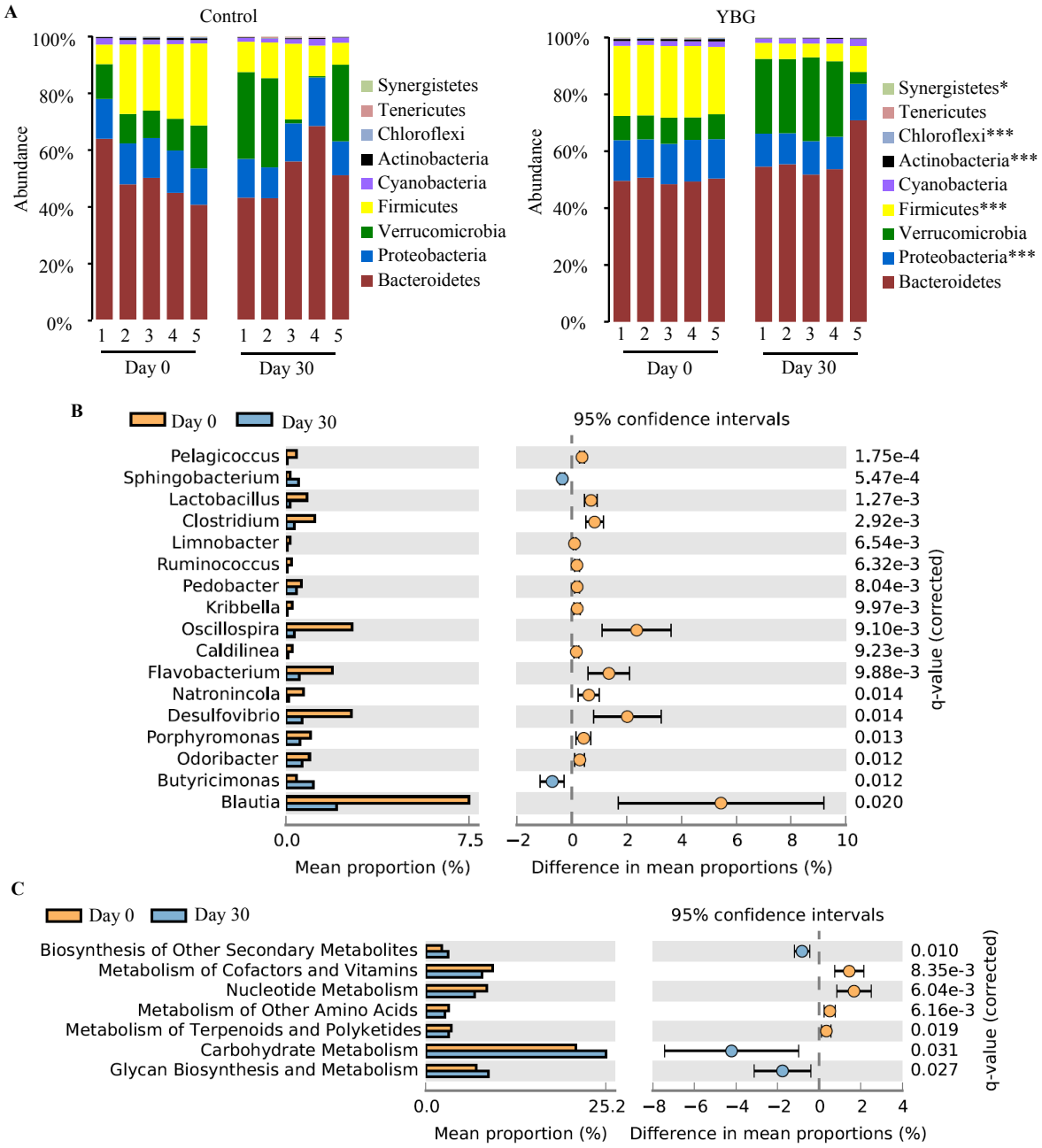

A
Control
YBG
Abundance
100%
80%
60%
40%
20%
0%
1 2 3 4 5
Day 0
Day 30
Synergistetes
Tenericutes
Chloroflexi
Actinobacteria
Cyanobacteria
Firmicutes
Verrucomicrobia
Proteobacteria
Bacteroidetes
Synergistetes*
Chloroflexi***
Actinobacteria***
Firmicutes***
Proteobacteria***
B
Day 0
Day 30
95% confidence intervals
Pelagicoccus 1.75e-4
Sphingobacterium 5.47e-4
Lactobacillus 1.27e-3
Clostridium 2.92e-3
Limnobacter 6.54e-3
Ruminococcus 6.32e-3
Pedobacter 8.04e-3
Kribbella 9.97e-3
Oscillospira 9.10e-3
Caldilinea 9.23e-3
Flavobacterium 9.88e-3
Natronincola 0.014
Desulfovibrio 0.014
Porphyromonas 0.013
Odoribacter 0.012
Butyricimonas 0.012
Blautia 0.020
q-value (corrected)
0.0
7.5
Mean proportion (%)
−2 0 2 4 6 8 10
Difference in mean proportions (%)
C
Day 0
Day 30
95% confidence intervals
Biosynthesis of Other Secondary Metabolites 0.010
Metabolism of Cofactors and Vitamins 8.35e-3
Nucleotide Metabolism 6.04e-3
Metabolism of Other Amino Acids 6.16e-3
Metabolism of Terpenoids and Polyketides 0.019
Carbohydrate Metabolism 0.031
Glycan Biosynthesis and Metabolism 0.027
q-value (corrected)
0.0
25.2
Mean proportion (%)
−8 −6 −4 −2 0 2 4
Difference in mean proportions (%)

Fig. 4

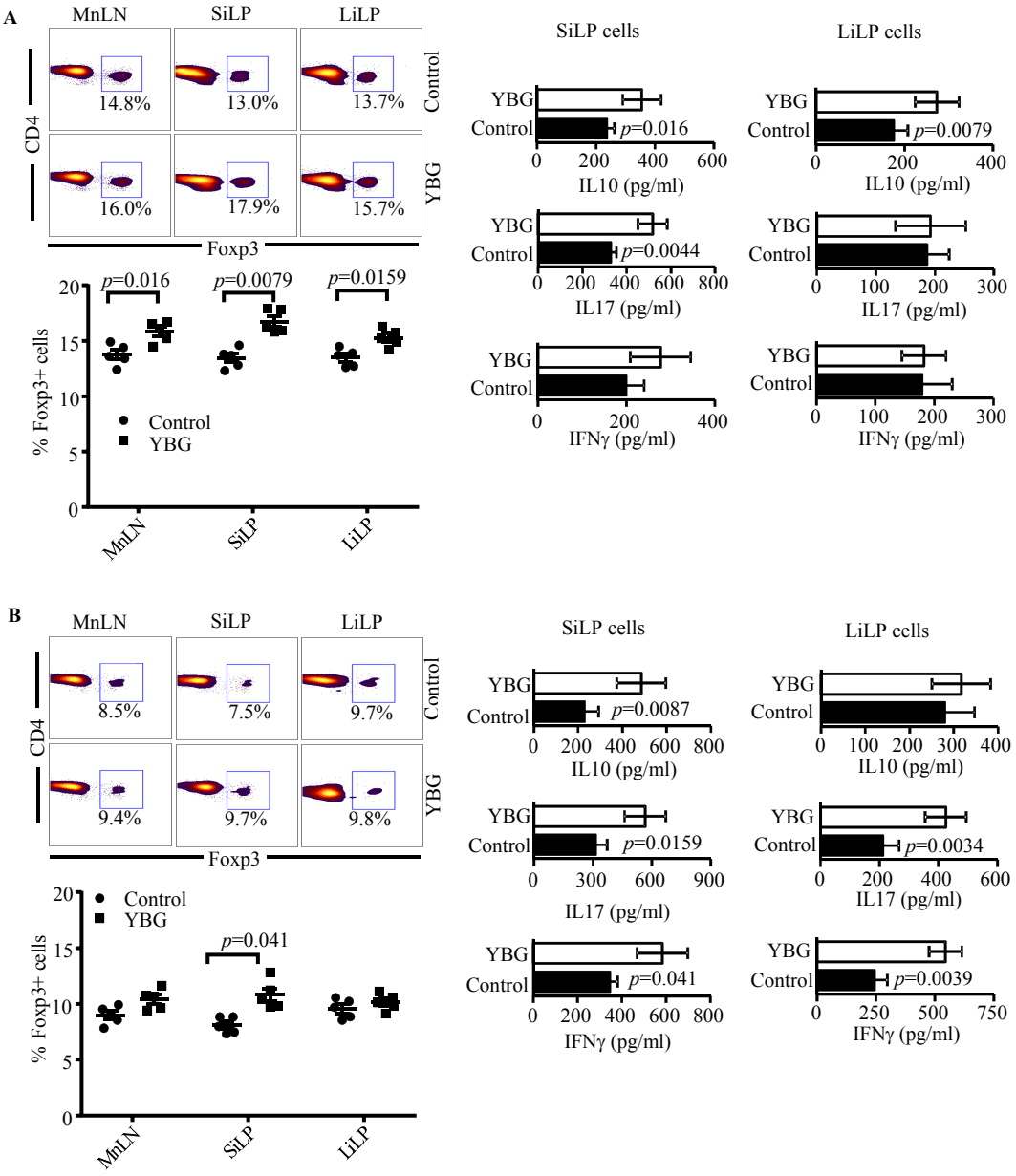

A
MnLN
SiLP
LiLP
CD4
Control
YBG
14.8%
13.0%
13.7%
16.0%
17.9%
15.7%
Foxp3
% Foxp3+ cells
p=0.016
p=0.0079
p=0.0159
Control
YBG
SiLP cells
LiLP cells
IL10 (pg/ml)
IL17 (pg/ml)
IFNγ (pg/ml)
p=0.016
p=0.0079
p=0.0044
B
8.5%
7.5%
9.7%
9.4%
9.7%
9.8%
p=0.041
p=0.0087
p=0.0159
p=0.0034
p=0.0039

Fig. 5

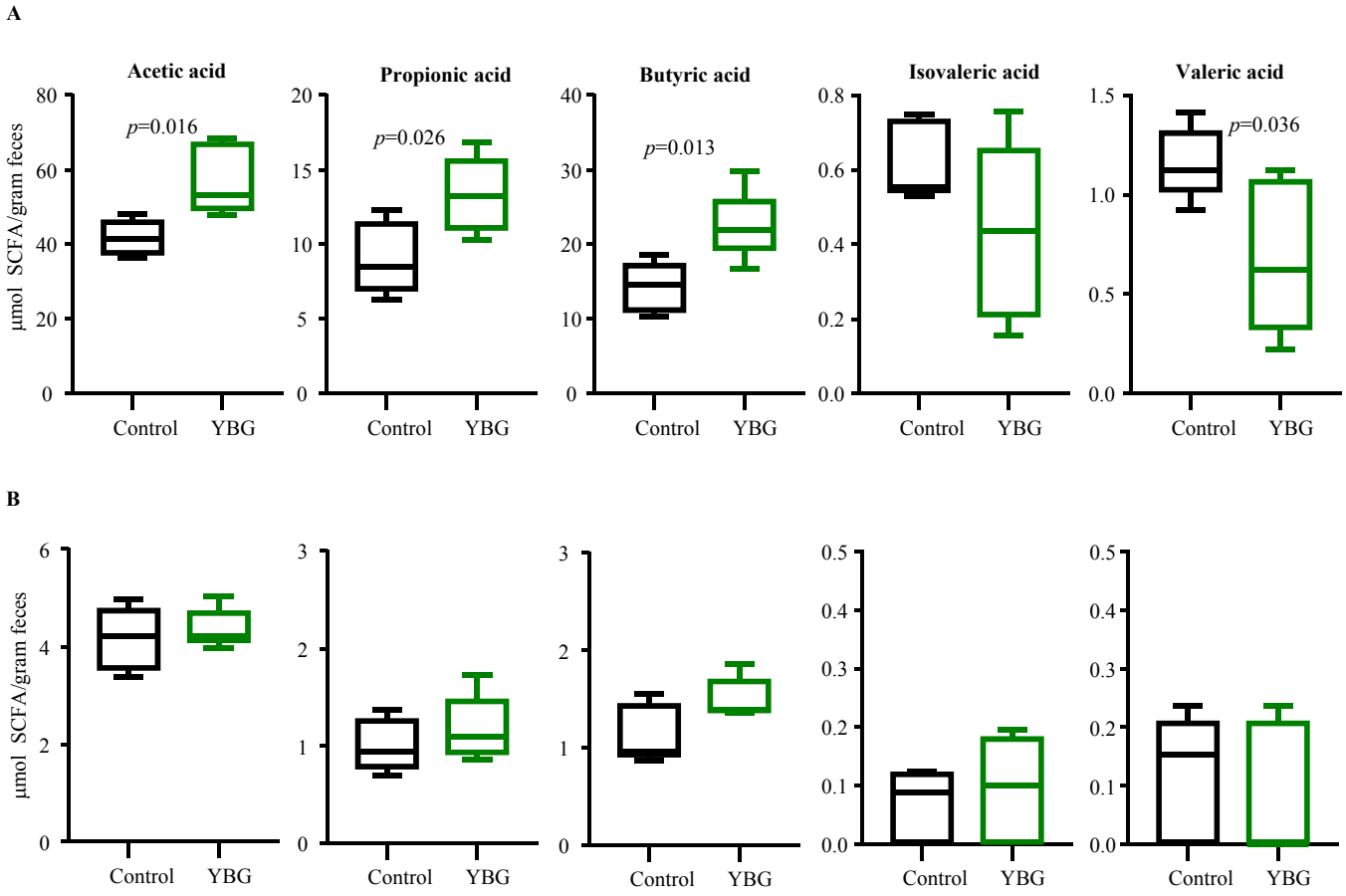
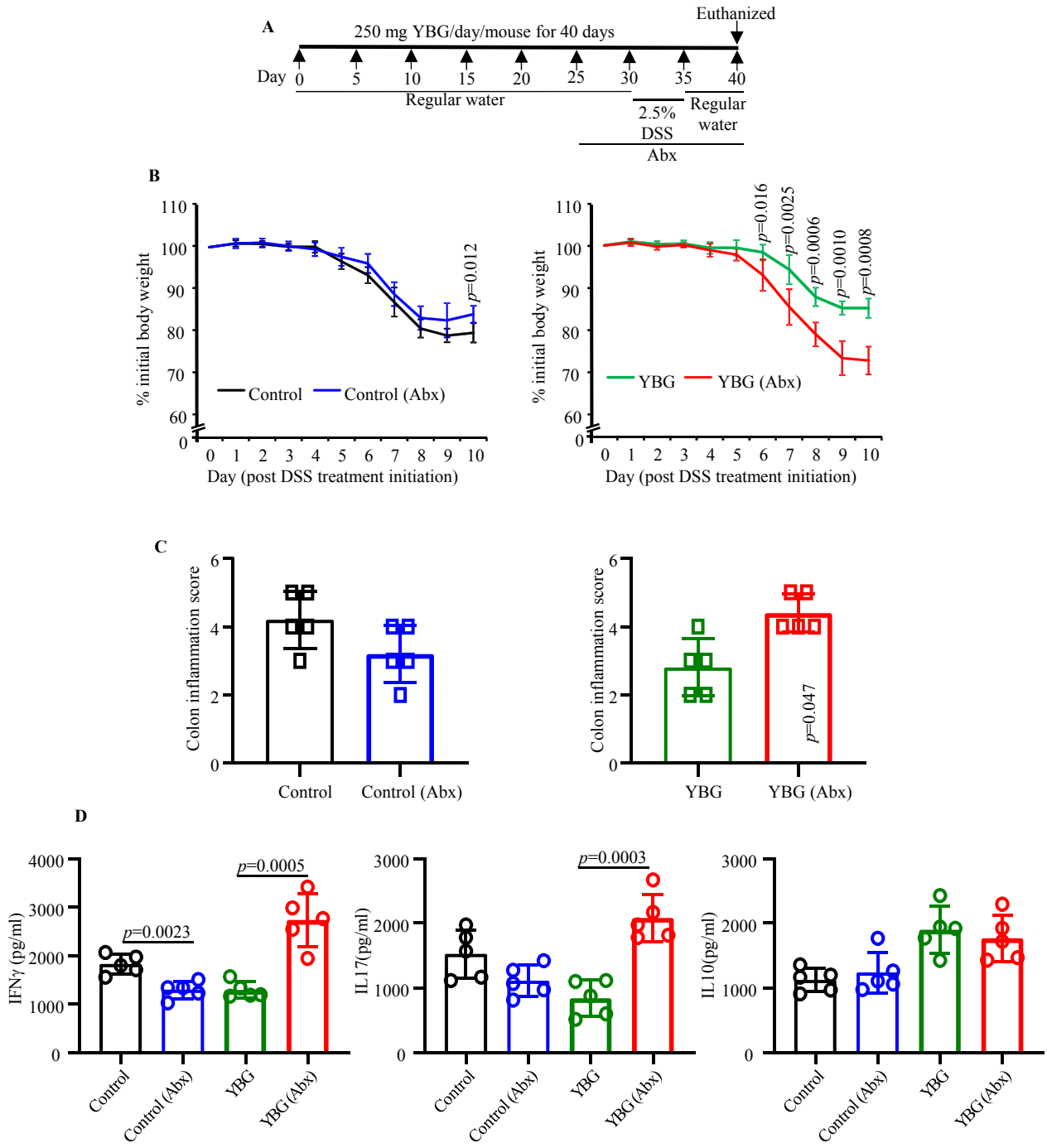

A
Acetic acid
Propionic acid
Butyric acid
Isovaleric acid
Valeric acid
μmol SCFA/gram feces
p=0.016
p=0.026
p=0.013
p=0.036
Control
YBG
B
μmol SCFA/gram feces
Control
YBG

Fig. 6


A
250 mg YBG/day/mouse for 40 days
Euthanized
Day 0 5 10 15 20 25 30 35 40
Regular water
2.5% DSS
Regular water
Abx
B
% initial body weight
Control
Control (Abx)
p=0.012
Day (post DSS treatment initiation)
YBG
YBG (Abx)
p=0.016
p=0.0025
p=0.0006
p=0.0010
p=0.0008
C
Colon inflammation score
Control
Control (Abx)
YBG
YBG (Abx)
p=0.047
D
IFNγ (pg/ml)
p=0.0023
p=0.0005
IL17(pg/ml)
p=0.0003
IL10(pg/ml)
Control
Control (Abx)
YBG
YBG (Abx)