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Administration of *Aspergillus oryzae* suppresses DSS-induced colitis

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ABSTRACT

Aspergillus oryzae, a filamentous fungus, has long been used for the production of traditional Japanese foods. Here, we analyzed how *A. oryzae* administration affects the intestinal environment in mice. The results of 16S rRNA gene sequencing of the gut microbiota indicated that after the administration of heat-killed *A. oryzae* spores, the relative abundance of an anti-inflammatory *Bifidobacterium pseudolongum* strain became 2.0-fold greater than that of the control. Next, we examined the effect of *A. oryzae* spore administration on the development of colitis induced by dextran sodium sulfate in mice; we found that colitis was alleviated by not only heat-killed *A. oryzae* spores, but also the cell wall extracted from the spores. Our findings suggest that *A. oryzae* holds considerable potential for commercial application in the production of both traditional Japanese fermented foods and new foods with prebiotic functions.

1. Introduction

Prebiotics are non-digestible food ingredients that enhance host health by stimulating the growth and/or activity of certain groups of beneficial bacteria in the gut (Gibson et al., 2004). For example, polysaccharides present in dietary fibers, such as pectin, β-(1,3)-glucan, β-(1,3)-/-β-(1,6)-glucan, and chitin, act as prebiotics (Jayachandran et al., 2018; Sanders et al., 2019), and *Bacteroides*, *Bifidobacterium*, and *Lactobacillus* species exhibit a strong ability to decompose these polysaccharides by producing various polysaccharide-degrading enzymes. The bacteria convert polysaccharides into oligosaccharides and monosaccharides, which are then metabolized to various metabolites, such as short-chain fatty acids (Ndeh and Gilbert, 2018; Ndeh et al., 2017). Notably, the short-chain fatty acids produced by *Bacteroides*, *Bifidobacterium*, and *Lactobacillus* species are associated with certain benefits, such as improvement of the intestinal barrier function, induction of anti-obesity effects, and enhancement of the immune system (Nicholson et al., 2012).

The human gut microbiome profoundly influences host health by affecting various intestinal functions (Bäumler and Sperandio, 2016; Sánchez et al., 2017). Recently, the diversity of the gut microbiome among different human populations was suggested to be influenced by geographical, cultural, and traditional differences (Lawley et al., 2019; Rubel et al., 2020). For example, among the gut microbes in Japanese people, the *Bifidobacterium* and *Blautia* populations were found to be large, whereas the *Clostridium* population was found to be small (Nakayama et al., 2015; Nishijima et al., 2016), and the *Bifidobacterium* population, in particular, was found to be larger in the gut microbiome of Japanese people than in that of people from other countries (Nakayama et al., 2015; Nishijima et al., 2016). Therefore, the high abundance of *Bifidobacterium* could potentially result from the consumption of various traditional and unique Japanese foods. However, the nutrients in traditional Japanese foods that contribute to the abundance of *Bifidobacterium* species remain to be elucidated.

Recently, UNESCO registered Washoku, a traditional Japanese cuisine, as an intangible cultural heritage. In Japan, over the past 1000 years, the filamentous fungus *Aspergillus oryzae* has been used for the production of sake, miso, soy sauce, amazake, and other traditional

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fermented beverages (awamori and shochu) (Ichishima, 2016). The consumption of *A. oryzae* is generally regarded to be safe by the US Food and Drug Administration, and the fungus has been used for producing fermented foods, organic acids, beneficial secondary metabolites, and various enzymes. Thus, Japanese fermented foods contain various organic acids, beneficial secondary metabolites, proteins, and enzymes produced by the fungus, and several polysaccharides that are components of the fungal cell wall, such as β-(1,3)-glucan, β-(1,3)-/-β-(1,6)-glucan, chitin, and galactosaminogalactan (Beauvais et al., 2014; Gow et al., 2017; Yoshimi et al., 2016). Intriguingly, fungal cell-wall polysaccharides, such as β-(1,3)-glucan, β-(1,3)-/-β-(1,6)-glucan, and galactosaminogalactan, have recently been reported to alleviate dextran sodium sulfate (DSS)-induced colitis in mice (Briard et al., 2020; Sun et al., 2019). These findings suggest that *A. oryzae* metabolites, proteins, and/or cell wall components present in foods can favorably affect host health. Here, we analyzed the effects of *A. oryzae* administration on the intestinal environment in mice.

2. Material and methods

2.1. Fungal strain, culture, and medium

The *A. oryzae* strain RIB40 was obtained from NBRC (http://www.nbrc.nite.go.jp) and cultured on Czapek-Dox agar (pH 6.0) at 30 °C (Hata et al., 1992). After cultivation for 3 days, spores were collected in saline.

2.2. Cell wall preparation from A. oryzae

A. oryzae RIB40 was cultured in Czapek-Dox liquid medium for 3 days, as described in Section 2.1. After cultivation, *A. oryzae* spores were collected in 50 mL tubes and centrifuged at 3500*g* for 10 min at 4 °C, following which the supernatant was discarded, and the pellet was resuspended in 30 mL of 0.1 mol/L sodium phosphate buffer (pH 6.0) and incubated at 120 °C for 60 min. The sample was cooled and then centrifuged at 3500*g* for 10 min at 4 °C, the supernatant was discarded,
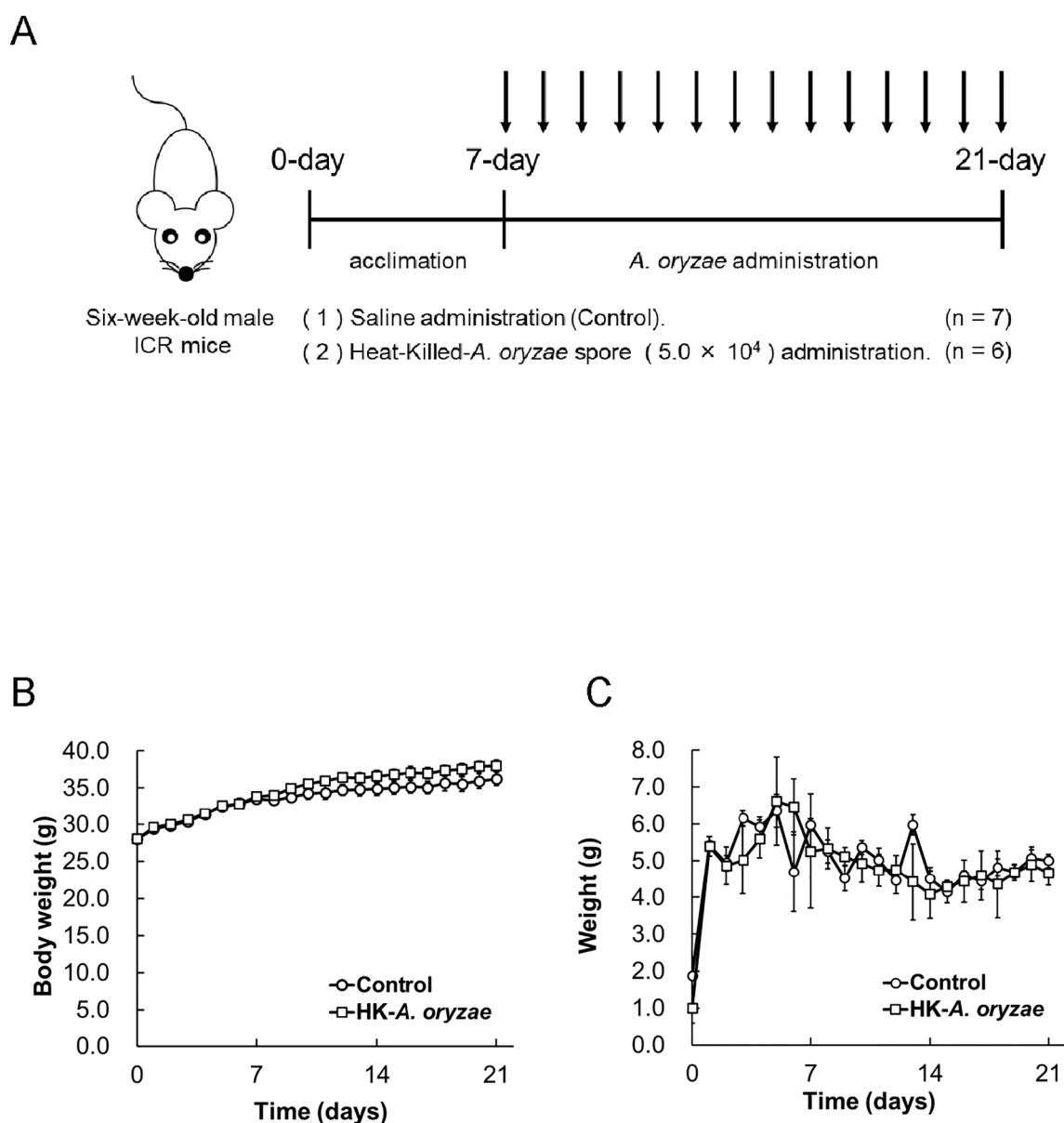

Fig. 1. Effect of *Aspergillus oryzae* administration on the body weight and food intake of mice. (A) Experimental protocol. Mice were treated daily with either heat-killed (HK) *A. oryzae* spores (n = 6) or saline (n = 7) until the end of the experiment. (B) Body weight changes. (C) Food intake. Data are presented as means ± standard error (error bars).

and the pellet was resuspended in a 0.5 mol/L NaOH solution. After incubation at 40 °C for 12 h, the sample was again centrifuged at 3500*g* for 10 min at 4 °C, the supernatant was discarded, and the pellet was resuspended in a 2 mol/L NaOH solution. Lastly, after incubation at 4 °C for 12 h, the sample was centrifuged at 3500*g* for 10 min at 4 °C, the supernatant was discarded, and the pellet was resuspended in saline, dialyzed, and freeze-dried.

2.3. Animals

Six-week-old male ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were housed individually with free access to food (AIN-93G) and drinking water and maintained on a 12 h/12 h light/dark cycle (lights on at 07:00 and off at 19:00) at 25 °C and 50% humidity. At the end of the experiment, the mice were sacrificed by over-anesthetization with isoflurane (FUJIFILM Wako, Osaka, Japan). This study was approved by the Institutional Animal Care and Use Committee of Meijo University (No. 2021AE06) and carried out in accordance with the guidelines of Meijo University Animal experiments.

2.4. Administration of A. oryzae spores to mice

Six-week-old male ICR mice were acclimated for 7 days and then separated into two groups: (1) control mice (n = 7), and (2) mice administered heat-killed (70 °C, 30 min) *A. oryzae* spores (0.13 mg; 5.0 $\times$ 10^4 cells/100 μL) (n = 6). Heat-killed *A. oryzae* spores were administered once daily for 21 days (Fig. 1A). To analyze the gut bacterial composition using a metagenomic approach, feces were collected at 7 days after spore administration and immediately stored at −80 °C until use for the metagenomic analysis. The body weight and weight of the food consumed by mice (food intake) were assessed daily.

2.5. Metagenomic analysis of gut microbiota

Amplicon preparation for 16S rDNA analysis and sequencing using a MiSeq platform (Illumina, San Diego, CA, USA) were conducted by Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). Briefly, total genomic DNA was extracted from 70 mg of feces using the MPure Bacterial DNA Extraction Kit (MP Biomedicals, Tokyo, Japan) per the manufacturer's instructions, following which the DNA concentration was measured using a Synergy H1 reader (BioTek Instruments, Winooski, VT, USA) and a QuantiFluor dsDNA System (Promega Corp., Madison, WI, USA). The DNA library was prepared using the following 2-step tailed-PCR method: first, the V3–V4 region of the 16S rRNA gene was amplified using a bacterial universal primer set (1st-27F mod, 5′-ACACTCTTTC CCTA-CACGAC GCTCTTCCGA TCT-AGRGTTT GATYMTGGCT CAG-3′, and 1st-338R, 5′-GTGACTGGAG TTCAGACGTG TGCTCTTCCG ATCT-TGCTGC CTCCCGTAGG AGT-3′; or 1st-27F mod_MIX, 5′-ACACTCTTTC CCTACACGAC GCTCTTCCGA TCT-NNNNN-AG RGTTTGATYM-TGGCT CAG-3′, and 1st-338R_MIX, 5′-GTGACTGGAG TTCAGACGTG TGCTCTTCCG ATCT-NNNNN-T GCTGCCTCCC GTAG-GAGT-3′). Next, the amplicon was purified, and a second PCR was performed using the following primer set: 2ndF, 5′-AATGATACGG CGACCACCGA GATCTACAC-Index2-ACACTCTTTC CCTACACGAC GC-3′, and 2ndR, 5′-CAAGCAGAAG ACGGCATACG AGAT-Index1-GTGACTGGAG TTCAGACGTG TG-3′. After amplicon purification, the products were quantified using the Synergy H1 reader and the Quanti-Fluor dsDNA System, and the quality of the prepared libraries was confirmed using a Fragment Analyzer System with a dsDNA 915 Reagent Kit (Advanced Analytical Technologies, Inc., Ankeny, IA, USA). The libraries were sequenced using an Illumina MiSeq platform (300 bp, paired-end reads) at Bioengineering Lab. Co., Ltd.

The obtained raw FASTQ files were processed using the microbiome package QIIME 2 (version 2021.4) (https://qiime2.org). Reads were filtered using a quality score of 20 and dereplicated using q2-dada2. The representative sequence sets obtained using the quality-control

procedure were subjected to diversity and taxonomy analyses. In the taxonomy analysis, the representative sequences were classified into taxonomic groups using the Greengenes 16S rRNA gene database.

2.6. Administration of A. oryzae spores to mice with DSS-induced colitis

As described in Section 2.4-6-week-old male ICR mice were acclimated for 7 days and then administered 2.5% DSS (36–50 kDa; MP Biomedicals, lllkirch, France) in drinking water for 7 days to induce acute colitis. The mice used in this experiment were separated into four groups: (1) mice without DSS treatment (n = 16), (2) mice with DSS treatment (n = 16), (3) DSS-treated mice administered heat-killed *A. oryzae* spores (0.13 mg; 5.0 × 10^4 cells/100 μL) (n = 15), and (4) DSS-treated mice administered the cell wall of *A. oryzae* spores (0.10 mg/100 μL) (n = 15). The administration of fungal spores or cell wall was continued for 14 days (from Day 8 to Day 26), and the body weights of the mice were measured daily. The mice were sacrificed on the 26th day, and colon and spleen tissues were collected. To evaluate the damage caused by DSS-induced colitis, the colon lengths were measured and colon tissues were stained with hematoxylin and eosin (H&E) (Feldman and Wolfe, 2014). Colon tissues fixed in 4% paraformaldehyde solution were embedded in paraffin, sectioned (thickness = 4 μm), and stained using H&E according to the manufacturer's instructions.

2.7. Quantification of inflammatory cytokines

The blood samples of the mice were collected and centrifuged at 1000*g* for 15 min at 4 °C. The supernatant was separated and stored at −80 °C for measurement. The plasma concentrations of inflammatory cytokines (IL-6 and IL-1β) were determined using enzyme-linked immunosorbent assay kits (Cosmo Bio Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions.

2.8. Quantitative PCR

The total DNA of the microbial population present in the feces samples was extracted using a QIAamp Fast DNA Stool Mini Kit (QIA-GEN, Hilden, Germany). The 16S rRNA gene of bifidobacteria was amplified using the following universal primers: Bifidobacteria F, 5′-AGGGTTCGATTCTGCTCAG-3′, and Bifidobacteria R, 5′-CATCCGG-CATTACCACCC-3′ (Ahmed et al., 2007). Each amplification reaction mixture (25 μL) contained 12.5 μL of 2 × TB Green Premix Ex Taq II (TaKaRa Bio Inc., Shiga, Japan), 1 μL of forward and reverse primers (10 μmol/L), 2 μL of bacterial DNA (10 ng), and 8.5 μL of ddH$_2$O. The following PCR protocol was used: initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 62 °C for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The relative abundance of the 16S rRNA gene of bifidobacteria was normalized against that of total bacteria.

2.9. Quantification of A. oryzae glycosylceramide in fermented foods

To determine the *A. oryzae* contents in commercial fermented foods, such as komekoji, amazake, and shiokoji, we quantified glycosylceramide extracted from *A. oryzae* (Ferdouse et al., 2019). Spores (10–100 mg) from cultures grown on Czapek-Dox agar plates (pH 6.0) were collected and lyophilized, and the fermented foods (3.0 g) were also lyophilized. The spores and the fermented foods were aseptically disrupted using liquid nitrogen in a mortar and pestle, and the fine powder obtained was dissolved in 2 mL of chloroform:methanol (1:1, v/v) and vortexed for 5 min. After sonicating the solution for 1 h, 8 mL of methanol containing 0.8 mol/L KOH was added, and the solution was incubated at 42 °C for 30 min, following which 20 mL of chloroform and 9 mL of distilled water were added, and the mixture was vortexed until saponification. After centrifugation at 800*g* for 10 min at 25 °C, the lower phase was recovered, evaporated under vacuum, and dissolved in

200 μL of chloroform:methanol (2:1, v/v). An aliquot of the resultant solution was spotted onto a thin-layer chromatography (TLC) plate (Silica gel 60 plate, Merck Millipore Inc., Darmstadt, Germany) and, subsequently, the dried plate was developed using a saturated chloroform:methanol:acetic acid:water solution (20:3.5:2.3:0.7, v/v). As a standard, 10 μL of 0.1–10.0 mg/mL cerebroside (Matreya Inc., Pleasant Gap, PA, USA) was spotted onto the TLC plates. To detect glycosylceramide, the TLC plates were sprayed with 2 mg/mL orcinol (MP

Biomedicals) in 15% sulfuric acid and heated at 100 °C for 5 min. The intensities and areas of the glycosylceramide spots on the TLC plates were determined and quantified using ImageJ software. Glycosylceramide from *A. oryzae* was analyzed using MALDI-TOF-MS, as previously described (Hirata et al., 2012).
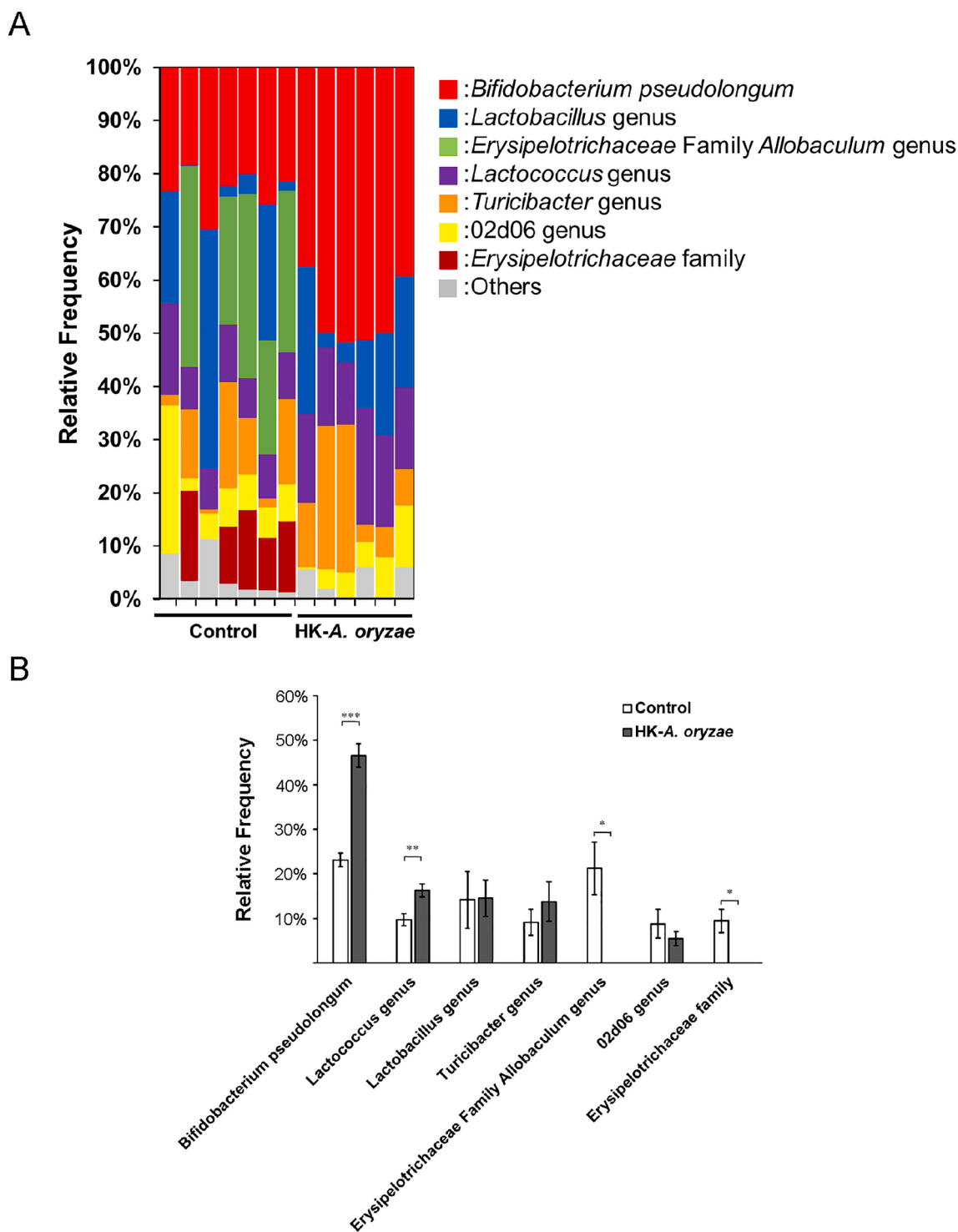

Fig. 2. Effect of *Aspergillus oryzae* administration on gut microbiota. (A) Taxonomy bar plot at species level. Biological replicates (6 or 7 replicates in each group) are displayed in separate bars. (B) Relative abundance of major taxa detected among the intestinal microbiota of mice. Error bars: standard error (n = 6 or 7). Differences were assessed using Welch's *t*-test (*$0.01 < p < 0.05$, **$0.001 < p < 0.01$, ***$p < 0.001$).

3. Results

3.1. The administration of A. oryzae spores affects gut microbiota in mice

We investigated how the administration of heat-killed *A. oryzae* spores affects the body weight and food intake (weight of diet consumed) of mice. The body weight and food intake of mice were not altered in response to the administration of *A. oryzae* spores (Fig. 1B and C).

Next, we determined the effects of *A. oryzae* spore administration on the structure and constitution of the gut microbial community (Fig. 2 and Supplementary Table 1). Feces were collected from control mice and mice treated with *A. oryzae* spores, and the V3–V4 region of the 16S rRNA gene isolated from microbes present in the feces was sequenced using the Illumina MiSeq platform; following the removal of unqualified sequences, we obtained 332,902 reads in total and an average of 25,608 reads (standard deviation = 5412) per sample. After the 7-day administration of the spores, the bacterial compositions of the gut microbiota in the two groups were compared (Fig. 2 and Supplementary Table 1). At the phylum level, the population of Actinobacteria in the gut microbiota of mice treated with heat-killed *A. oryzae* spores was larger than that of the control, whereas the relative abundance of Firmicutes was lower (Supplementary Fig. 1). To further investigate the differences among the groups, the bacterial taxa were identified (Fig. 2A and B). After the administration of heat-killed *A. oryzae* spores, *Bifidobacterium pseudolongum*, from the phylum Actinobacteria, was found to be relatively more abundant, with a population 2.0-fold larger than that in the control and accounting for 40% of the total bacterial population (Fig. 2A and B). Conversely, the relative abundances of family *Erysipelotrichaceae* and family *Erysipelotrichaceae* genus *Allobaculum* were significantly lower than those in the control, although genus *Lactococcus* was more abundant after spore administration (Fig. 2A and B). These results indicate that mice treated with heat-killed *A. oryzae* spores exhibited a distinct bacterial composition compared with mice that were not treated with *A. oryzae* spores.

3.2. The administration of A. oryzae spores alleviates DSS-induced colitis in mice

We examined the effects of *A. oryzae* spore administration on the development of DSS-induced colitis in mice. Mice were continuously administered *A. oryzae* spores for 7 days before DSS treatment, following which colitis was induced by including 2.5% DSS in drinking water for 7 days (Fig. 3A); the *A. oryzae* spores were administered daily until the end of the experiment (Fig. 3A). The body weight and colon length of DSS-treated mice decreased by 19.2% and 31.0%, respectively, as compared with the corresponding measurements in non-treated mice (Fig. 3B, C, and D). Next, H&E staining was used to evaluate tissue damage in the mouse colon. In control mice, the colon exhibited normal morphology, characterized by an intact mucosa, abundance of goblet cells, and thin muscularis and submucosa layers; in contrast, in DSS-treated mice, the colon exhibited severe damage, including major damage to the epithelial surface, colonocyte loss, goblet cell exhaustion, crypt distortion, myometrial edema and thickening, mucosal inflammation, and extensive inflammatory cell infiltration (Supplementary Fig. 2). Notably, the administration of *A. oryzae* spores attenuated the pathological changes induced by DSS, which helped maintain an intact colon tissue morphology (Fig. 3B, C, and D and Supplementary Fig. 2). These results suggest that the administration of *A. oryzae* spores reduced DSS-induced damage in colon tissues.

DSS treatment is known to increase spleen weight and size, which is generally correlated with the extent of inflammation and anemia (van Tilburg Bernardes et al., 2020; Yan et al., 2018). Here, DSS treatment increased the spleen weight and size, whereas the administration of *A. oryzae* spores suppressed DSS-induced spleen enlargement (Fig. 3E and F).

The role of pro-inflammatory cytokines in inflammatory bowel disease (IBD) pathogenesis has been studied extensively, with their dysregulation considered a major sign of colon damage during IBD development (Chi et al., 2018; Neurath, 2014). In addition, pro-inflammatory cytokines, including IL-6 and IL-1β, have been shown to enhance paracellular permeability (Chang et al., 2021). Accordingly, we measured the levels of these cytokines in the plasma of colitis-induced mice to evaluate the anti-inflammatory effects of *A. oryzae* spore administration. As shown in Supplementary Fig. 3, the plasma IL-6 and IL-1β levels increased significantly in DSS-treated mice (to levels approximately 4–5-fold higher than those in controls). This indicated that inflammatory responses mounted after DSS treatment. Conversely, mice that were administered *A. oryzae* spores showed significantly lower pro-inflammatory cytokine levels. These results suggest that cytokine regulation in response to the administration of *A. oryzae* spores is conducive to the alleviation of inflammatory responses, which helps preserve the intestinal barrier integrity.

Various polysaccharides have been reported to function as prebiotics and increase the population of *Bifidobacterium* species in the gut microbiota (Ma et al., 2021; Zheng et al., 2018). Intriguingly, the polysaccharides β-(1,3)-glucan, β-(1,3)-/-β-(1,6)-glucan, and chitin are known to possess anti-inflammatory properties (Jayachandran et al., 2018; Sun et al., 2019; Wagener et al., 2014), and the fungal cell wall has been shown to contain these polysaccharides (Beauvais et al., 2014; Gow et al., 2017; Yoshimi et al., 2016). Thus, we examined how the administration of the cell wall of *A. oryzae* spores affects the development of DSS-induced colitis in mice. We found that treatment with spore cell wall suppressed colitis development (Fig. 3B and C). Moreover, in agreement with findings from a previous study (Jing et al., 2017), spleen enlargement was observed in DSS-treated mice, but not in mock-treated mice or DSS-treated mice that were also administered the spore cell wall (Fig. 3E and F). Mice administered the spore cell wall preparation exhibited a significantly low IL-1β level, and the IL-6 level also showed a decreasing trend (Supplementary Fig. 3). These results indicate that polysaccharides present in the cell wall of *A. oryzae* partially alleviate DSS-induced colitis.

We next used quantitative PCR to ascertain how the *B. pseudolongum* population in the gut microbiota is affected after spore cell wall treatment (Fig. 4). As observed after the 7- and 14-day administration of *A. oryzae* spores, *B. pseudolongum* was relatively more abundant after the administration of the spore cell wall, as the population was 2.0-fold greater than that in the control (Fig. 4). These results indicate that the polysaccharides in the fungal cell wall partially function as prebiotics.

3.3. A. oryzae contents in Japanese fermented foods

Lastly, the *A. oryzae* content in commercially available fermented foods was determined based on the glycosylceramide concentrations (Supplementary Fig. 4). Glycosylceramide extracted from *A. oryzae* spores and the Japanese fermented food shiokoji was analyzed on TLC plates and identified using MALDI-TOF-MS (Supplementary Fig. 4). The main peak was consistent with the mass of glycosylceramide (m/z 776.6) (Supplementary Fig. 4D). A similar spectrum of glycosylceramide has been reported previously (Hirata et al., 2012). The glycosylceramide content was 4.6 μg/10 mg of spores (Supplementary Fig. 4A and 4B). Next, the glycosylceramide contents of the Japanese fermented foods shiokoji, amazake, and pulverized komekoji A/B/C were measured (Supplementary Fig. 4C) and found to be 25, 8, and 156/128/202 μg in 1.0 g of the foods, respectively (Table 1 and Supplementary Fig. 4C); thus, the glycosylceramide content was higher in komekoji than in the other fermented foods (Table 1). We also calculated the *A. oryzae* content in commercially available fermented foods based on the glycosylceramide content, and we found that 1.0 g of shiokoji, amazake, and komekoji A/B/C contained 53, 17, and 330/270/430 mg of *A. oryzae*, respectively.
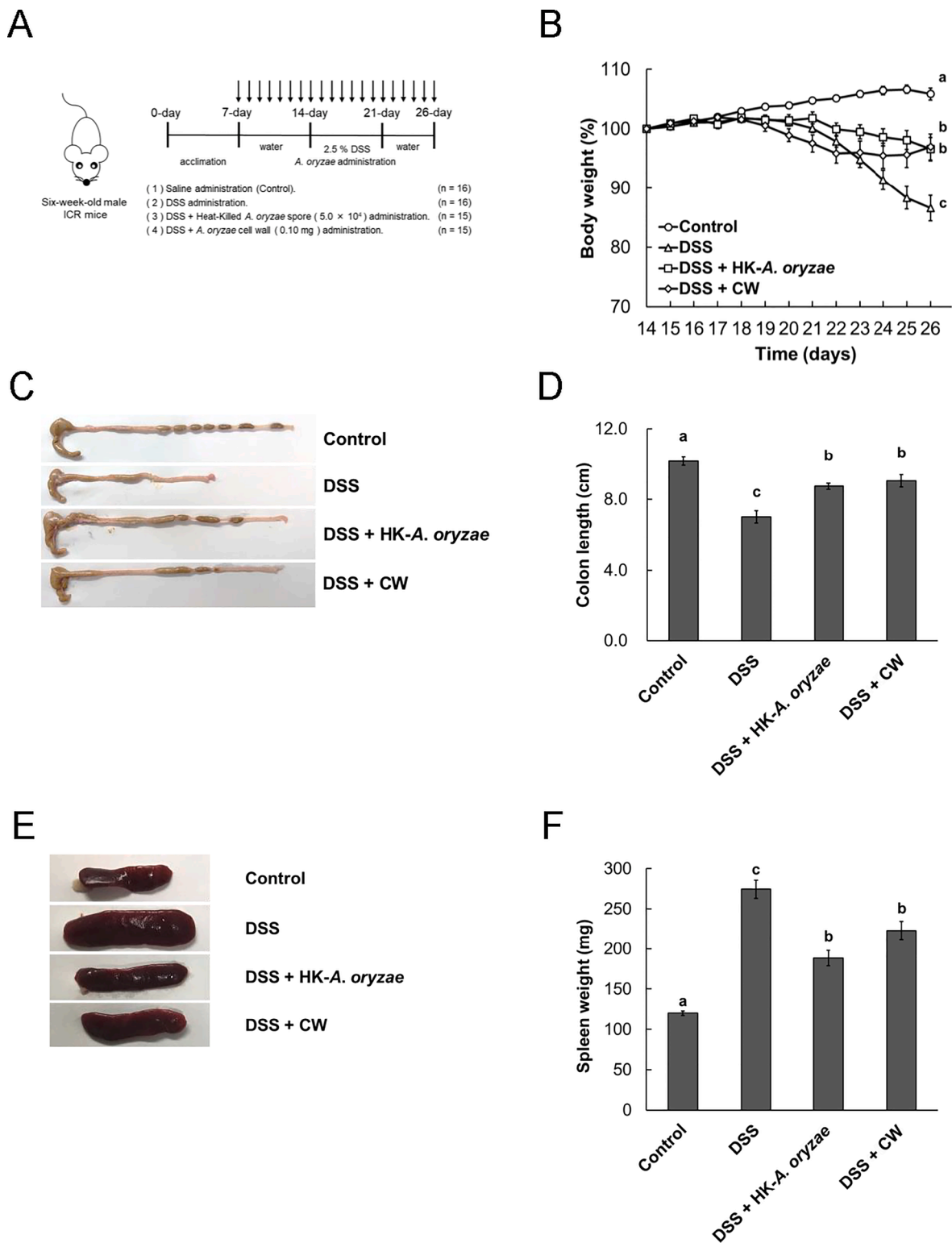
Fig. 3. Effect of *Aspergillus oryzae* administration on dextran sodium sulfate (DSS)-induced colitis. (A) Mice were separated into four groups: (1) mice without DSS treatment (n = 16), (2) mice with DSS treatment (n = 16), (3) DSS-treated mice administered heat-killed *A. oryzae* spores (n = 15), and (4) DSS-treated mice administered *A. oryzae* spore cell wall (n = 15). Colonic inflammation was induced by the 7-day administration of 2.5% (w/v) DSS in drinking water. (B) Mouse body weight was evaluated throughout the experiment, and values are presented as percentages of change relative to the initial value measured before DSS administration. Different letters represent statistically significant differences ($p < 0.05$). (C, D) Colon length. Colon length was evaluated at the end of the experiment. Different letters represent statistically significant differences ($p < 0.05$). (E, F) Spleen weight. Spleen weight was evaluated at the end of the experiment. Different letters represent statistically significant differences ($p < 0.01$). Data are presented as means ± standard error (error bars). Differences were assessed using Tukey's HSD test with Box-Cox transformed data.
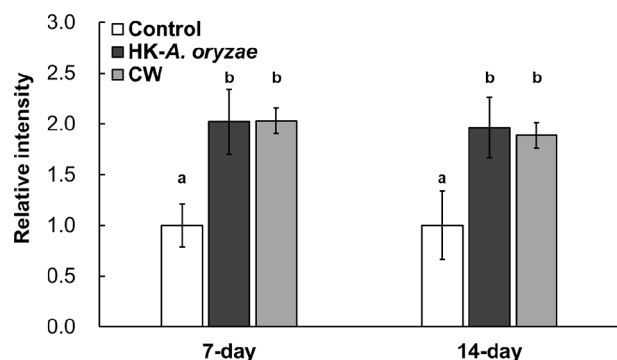
Fig. 4. Effect of cell wall administration on the *Bifidobacterium pseudolongum* population. Quantitative PCR was used to measure the *B. pseudolongum* population in mouse gut microbiota after the administration of *Aspergillus oryzae* spore cell wall. Mice were treated daily with heat-killed (HK) *A. oryzae* spores (n = 5), *A. oryzae* spore cell wall (n = 5), or saline (n = 5), as described in Fig. 1A. The abundance of amplified fragments of 16S DNA from *B. pseudolongum* was normalized relative to the abundance of amplified fragments of total 16S DNA in the feces samples. Data are presented as means ± standard error (error bars) from five independent experiments. Differences were assessed using Tukey's HSD test with Box-Cox transformed data. Different letters represent statistically significant differences ($p < 0.01$).

Table 1
Quantification of *A. oryzae* in Japanese fermented foods.

Foods	Glycosylceramide (μg/1 g food)	*A. oryzae* (mg/1 g food)	Estimated intake (g/day)
Shiokoji	25 ± 2	53 ± 4	3.8
Amazake	8 ± 1	17 ± 1	11.5
Komekoji A	156 ± 5	330 ± 11	0.6
Komekoji B	128 ± 19	271 ± 4	0.7
Komekoji C	202 ± 18	430 ± 39	0.5

A. oryzae contents and estimated daily intake for humans (weight = 60.0 kg) were calculated from glycosylceramide contents in Japanese fermented foods.

4. Discussion

In this study, we analyzed the effects of the administration of heat-killed *A. oryzae* spores on the intraintestinal environment of mice. After spore administration, the population of *B. pseudolongum* was 2.0-fold greater than that in control mice. Moreover, the administration of *A. oryzae* spores prevented weight loss and reduced the severity of colitis, suggesting that the fungal spores alleviated DSS-induced colitis in mice.

The population of *Bifidobacterium* spp. in the gut microbiome is larger in Japanese people than in people from other countries (Nakayama et al., 2015). Our study revealed that the administration of *A. oryzae*, which is used in the production of traditional Japanese fermented foods, increased the *Bifidobacterium* population in the mouse gut microbiota. Japanese fermented foods, such as shiokoji, amazake, and komekoji, contain *A. oryzae* and its cellular components. The fungal cell wall is primarily composed of chitin, β-(1,3)-glucan, β-(1,3)-/-β-(1,6)-glucan, galactosaminogalactan, and α-mannan (Beauvais et al., 2014; Gow et al., 2017; Yoshimi et al., 2016), and previous analyses have revealed that the cell wall composition does not differ quantitatively between *A. oryzae* spores and mycelia (Horikoshi and Iida, 1964). The aforementioned polysaccharides are known to promote the growth of *B. pseudolongum* in the gut (Ma et al., 2021, Zheng et al., 2018), which suggests that the polysaccharides present in the cell wall of *A. oryzae* spores and/or mycelia in Japanese fermented foods function as prebiotics. Here, we calculated the *A. oryzae* contents in commercially available fermented foods based on the glycosylceramide content, and we found that the foods contained the fungus in distinct quantities. When the mice in this study (weighing ~40 g) were administered *A. oryzae* spores (0.13 mg/day), the population of *Bifidobacterium* species expanded, and DSS-induced colitis was alleviated. These results indicate that the daily intake of 0.5 g of komekoji C might produce similar effects in humans (weighing ~60.0 kg). Thus, *A. oryzae* is highly suitable for application in the development of new prebiotics that can alleviate colitis.

Bifidobacterium species are found in the intestinal flora of animals, particularly mice and rats. Various polysaccharides have previously been reported to function as prebiotics and increase the population of *Bifidobacterium* species in the gut (Ma et al., 2021, Zheng et al., 2018). Here, the administration of *A. oryzae* spores or spore cell wall increased the abundance of *B. pseudolongum* in mice. Because the cell wall of *A. oryzae* is composed of the various aforementioned polysaccharides, *B. pseudolongum* uses the polysaccharides as a carbon source, which helps increase its abundance among the gut microbes. *Bifidobacterium* species produce various enzymes of the glycoside hydrolase family, and three *B. pseudolongum* strains have been registered in the Carbohydrate-Active enZYmes Database (Cantarel et al., 2009). These bacteria harbor various β-1,3-glucanases, β-1,6-glucanases, chitinases, and α-mannanases, which are involved in the degradation of the cell wall polysaccharides of *A. oryzae*. These findings indicate that the polysaccharides in the fungal cell wall function as prebiotics.

Unrestricted inflammation at intestinal sites leads to the malabsorption of nutrients, severe abdominal pain, and increased risk of colorectal cancer development (Dyson and Rutter, 2012; Balestrieri et al., 2020). β-(1,3)-glucan, β-(1,3)-/-β-(1,6)-glucan, and chitin possess anti-inflammatory properties (Jayachandran et al., 2018; Sun et al., 2019; Wagener et al., 2014), and dietary polysaccharides such as β-(1,3)-/-β-(1,6)-glucan exert beneficial effects against IBD by blocking dectin-1 downstream signaling (Tang et al., 2015.). Here, the administration of *A. oryzae* spores or cell wall extracted from the spores alleviated DSS-induced colitis in mice, suggesting that β-(1,3)-glucan, β-(1,3)-/-β-(1,6)-glucan, and/or chitin present in the cell wall directly reduced inflammation in intestinal epithelial cells. Recently, galactosaminogalactan present in the cell wall of *A. fumigatus* has also been shown to exert anti-inflammatory effects in mice in an inflammasome-dependent manner (Briard et al., 2020). As noted in the preceding paragraph, polysaccharides might alleviate colitis by increasing the population of *Bifidobacterium* species. However, it is currently unclear whether the polysaccharides ameliorate colitis by directly reducing inflammation by blocking dectin-1 downstream signaling and/or acting in an inflammasome-dependent manner, or by increasing the population of *Bifidobacterium* species.

The involvement of *Erysipelotrichaceae* in chronic intestinal inflammation has been reported previously (Park et al., 2020), and an imbalance between the *Erysipelotrichaceae* and *Lachnospiraceae* populations in the gut microbiota has been reported to cause IBD (Chen et al., 2017). We found here that the relative abundances of family *Erysipelotrichaceae* and family *Erysipelotrichaceae* genus *Allobaculum* were markedly decreased in mice that were administered heat-killed *A. oryzae* spores. These findings suggest that the low levels of colitogenic strains of *Erysipelotrichaceae* resulting from the administration of *A. oryzae* spores are potentially associated with the reduction of DSS-induced damage to colon tissues.

5. Conclusion

We have shown here that *A. oryzae* used in the production of Japanese fermented foods functions as a prebiotic, and that the administration of either *A. oryzae* spores or the extracted spore cell wall alleviates DSS-induced colitis in mice. These findings suggest that *A. oryzae* could be useful for commercial application in the production of not only traditional Japanese fermented foods, but also new foods with prebiotic

functions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2021.100063.

References

Ahmed, S., Macfarlane, G. T., Fite, A., McBain, A. J., Gilbert, P., & Macfarlane, S. (2007). Mucosa-associated bacterial diversity in relation to human terminal ileum and colonic biopsy samples. *Applied Environmental Microbiology, 73*(22), 7435–7442. https://doi.org/10.1128/aem.01143-07

Balestrieri, P., Ribolsi, M., Guarino, M. P. L., Emerenziani, S., Altomare, A., & Cicala, M. (2020). Nutritional aspects in inflammatory bowel diseases. *Nutrients, 12*(2), 372. https://doi.org/10.3390/nu12020372

Bäumler, A. J., & Sperandio, V. (2016). Interactions between the microbiota and pathogenic bacteria in the gut. *Nature, 535*(7610), 85–93. https://doi.org/10.1038/nature18849

Beauvais, A., Fontaine, T., Aimanianda, V., & Latgé, J. P. (2014). Aspergillus cell wall and biofilm. *Mycopathologia, 178*(5–6), 371–377. https://doi.org/10.1007/s11046-014-9766-0

Briard, B., Fontaine, T., Samir, P., Place, D. E., Muszkieta, L., Malireddi, R. K. S., … Kanneganti, T.-D. (2020). Galactosaminogalactan activates the inflammasome to provide host protection. *Nature, 588*(7839), 688–692. https://doi.org/10.1038/s41586-020-2996-z

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., & Henrissat, B. (2009). The carbohydrate-active EnZymes database (CAZy): An expert resource for Glycogenomics. *Nucleic Acids Research, 37*(Database issue), D233–8. https://doi.org/10.1093/nar/gkn663

Chang, Y., Deng, Q., Zhang, Z., Zhao, H., Tang, J., Chen, X., … Jia, G. (2021). Glucagon-like peptide 2 attenuates intestinal mucosal barrier injury through the MLCK/pMLC signaling pathway in a piglet model. *Journal of Cell Physiology, 236*(4), 3015–3032. https://doi.org/10.1002/jcp.v236.410.1002/jcp.30068

Chen, L., Wilson, J. E., Koenigsknecht, M. J., Chou, W.-C., Montgomery, S. A., Truax, A. D., … Ting, J.-Y. (2017). NLRP12 attenuates colon inflammation by maintaining colonic microbial diversity and promoting protective commensal bacterial growth. *Nature Immunology, 18*(5), 541–551. https://doi.org/10.1038/ni.3690

Chi, J. H., Seo, G. S., & Lee, S. H. (2018). Oregonin inhibits inflammation and protects against barrier disruption in intestinal epithelial cells. *International Immunopharmacology, 59*, 134–140. https://doi.org/10.1016/j.intimp.2018.04.006

Dyson, J. K., & Rutter, M. D. (2012). Colorectal cancer in inflammatory bowel disease: What is the real magnitude of the risk? *World Journal of Gastroenterology, 18*(29), 3839–3848. https://doi.org/10.3748/wjg.v18.i29.3839

Feldman, A. T., & Wolfe, D. (2014). Tissue processing and hematoxylin and eosin staining. *Methods in Molecular Biology, 1180*, 31–43. https://doi.org/10.1007/978-1-4939-1050-2_3

Ferdouse, J., Miyagawa, M., Hirano, M., Kitajima, Y., Inaba, S., & Kitagaki, H. (2019). A new method for determining the mycelial weight of the koji-mold *Aspergillus oryzae* by measuring its glycosylceramide content. *The Journal of General and Applied Microbiology, 65*(1), 34–38. https://doi.org/10.2323/jgam.2018.04.003

Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutrition Research Reviews, 17*(2), 259–275. https://doi.org/10.1079/nrr200479

Gow, N. A. R., Latge, J.-P., Munro, C. A., & Heitman, J. (2017). The fungal cell wall: Structure, biosynthesis, and function. *Microbiology. Spectrum, 5*(3). https://doi.org/10.1128/microbiolspec.FUNK-0035-2016

Hata, Y., Kitamoto, K., Gomi, K., Kumagai, C., & Tamura, G. (1992). Functional elements of the promoter region of the *Aspergillus oryzae* glaA gene encoding glucoamylase. *Current Genetics, 22*(2), 85–91. https://doi.org/10.1007/bf00351466

Hirata, M., Tsuge, K., Jayakody, L. N., Urano, Y., Sawada, K., Inaba, S., … Kitagaki, H. (2012). Structural determination of glucosylceramides in the distillation remnants of shochu, the Japanese traditional liquor, and its production by *Aspergillus kawachii*. *Journal of Agricultural and Food Chemistry, 60*(46), 11473–11482. https://doi.org/10.1021/jf303117e

Horikoshi, K., & Iida, S. (1964). Studies of the spore coats of fungi I. Isolation and composition of the spore coats of *Aspergillus oryzae*. *Biochimica et Biophysica Acta (BBA) - Specialized Section on Mucoproteins and Mucopolysaccharides, 83*(2), 197–203. https://doi.org/10.1016/0926-6526(64)90035-7

Ichishima, E. (2016). Development of enzyme technology for *Aspergillus oryzae*, *A. sojae*, and *A. luchuensis*, the national microorganisms of Japan. *Bioscience, Biotechnology, and Biochemistry, 80*(9), 1681–1692. https://doi.org/10.1080/09168451.2016.1177445

Jayachandran, M., Chen, J., Chung, S. S. M., & Xu, B. (2018). A critical review on the impacts of β-glucans on gut microbiota and human health. *The Journal of Nutritional Biochemistry, 61*, 101–110. https://doi.org/10.1016/j.jnutbio.2018.06.010

Jing, W., Gao, X., Han, B., Wei, B., Hu, N., Li, S., … Wang, Y. (2017). Mori Cortex regulates P-glycoprotein in Caco-2 cells and colons from rats with experimental colitis via direct and gut microbiota-mediated mechanisms. *RSC Advances, 7*(5), 2594–2605. https://doi.org/10.1039/C6RA25448A

Lawley, B., Otal, A., Moloney-Geany, K., Diana, A., Houghton, L., Heath, A.-L., … Elkins, C. A. (2019). Fecal microbiotas of Indonesian and New Zealand children differ in complexity and bifidobacterial taxa during the first year of life. *Applied and Environmental Microbiology, 85*(19). https://doi.org/10.1128/AEM.01105-19

Ma, G., Hu, Q., Han, Y., Du, H., Yang, W., Pan, C., … Xiao, H. (2021). Inhibitory effects of β-type glycosidic polysaccharide from *Pleurotus eryngii* on dextran sodium sulfate-induced colitis in mice. *Food Function, 12*(9), 3831–3841. https://doi.org/10.1039/D0FO02905J

Nakayama, J., Watanabe, K., Jiang, J., Matsuda, K., Chao, S.-H., Haryono, P., … Lee, Y.-K. (2015). Diversity in gut bacterial community of school-age children in Asia. *Scientific Reports, 5*(1). https://doi.org/10.1038/srep08397

Ndeh, D., Rogowski, A., Cartmell, A., Luis, A. S., Baslé, A., Gray, J., … Gilbert, H. J. (2017). Corrigendum: Complex pectin metabolism by gut bacteria reveals novel catalytic functions. *Nature, 544*(7648), 65–70. https://doi.org/10.1038/nature23659

Ndeh, D., & Gilbert, H. J. (2018). Biochemistry of complex glycan depolymerisation by the human gut microbiota. *FEMS Microbiology Reviews, 42*(2), 146–164. https://doi.org/10.1093/femsre/fuy002

Neurath, M. F. (2014). Cytokines in inflammatory bowel disease. *Nature Reviews Immunology, 14*(5), 329–342. https://doi.org/10.1038/nri3661

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S. (2012). Host-gut microbiota metabolic interactions. *Science, 336*(6086), 1262–1267. https://doi.org/10.1126/science.1223813

Nishijima, S., Suda, W., Oshima, K., Kim, S. W., Hirose, Y., Morita, H., & Hattori, M. (2016). The gut microbiome of healthy Japanese and its microbial and functional uniqueness. *DNA Research, 23*(2), 125–133. https://doi.org/10.1093/dnares/dsw002

Park, Y. S., Kim, S. H., Park, J. W., Kho, Y., Seok, P. R., Shin, J.-H., … Kim, E. K. (2020). Melatonin in the colon modulates intestinal microbiota in response to stress and sleep deprivation. *Intestinal Research, 18*(3), 325–336. https://doi.org/10.5217/ir.2019.00093

Sánchez, B., Delgado, S., Blanco-Míguez, A., Lourenço, A., Gueimonde, M., & Margolles, A. (2017). Probiotics, gut microbiota, and their influence on host health and disease. *Molecular Nutrition Food Research, 61*(1), 1600240. https://doi.org/10.1002/mnfr.201600240

Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R., & Rastall, R. A. (2019). Probiotics and prebiotics in intestinal health and disease: From biology to the clinic. *Nature Reviews Gastroenterology & Hepatology., 16*(10), 605–616. https://doi.org/10.1038/s41575-019-0173-3

Sun, Y., Shi, X., Zheng, X., Nie, S., & Xu, X. (2019). Inhibition of dextran sodium sulfate-induced colitis in mice by baker's yeast polysaccharides. *Carbohydrate Polymers, 207*, 371–381. https://doi.org/10.1016/j.carbpol.2018.11.087

Tang, C.e., Kamiya, T., Liu, Y., Kadoki, M., Kakuta, S., Oshima, K., … Iwakura, Y. (2015). Inhibition of Dectin-1 signaling ameliorates colitis by inducing *Lactobacillus*-mediated regulatory T cell expansion in the intestine. *Cell Host & Microbe, 18*(2), 183–197. https://doi.org/10.1016/j.chom.2015.07.003

Rubel, M. A., Abbas, A., Taylor, L. J., Connell, A., Tanes, C., Bittinger, K., … Tishkoff, S. A. (2020). Lifestyle and the presence of helminths is associated with gut microbiome composition in Cameroonians. *Genome Biology, 21*(1). https://doi.org/10.1186/s13059-020-02020-4

van Tilburg Bernardes, E., Pettersen, V. K., Gutierrez, M. W., Laforest-Lapointe, I., Jendzjowsky, N. G., Cavin, J.-B., … Arrieta, M.-C. (2020). Intestinal fungi are causally implicated in microbiome assembly and immune development in mice. *Nature Communications, 11*(1). https://doi.org/10.1038/s41467-020-16431-1

Wagener, J., Malireddi, R. K. S., Lenardon, M. D., Köberle, M., Vautier, S., MacCallum, D. M., … Sil, A. (2014). Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. e1004050 *PLOS Pathogens, 10*(4). https://doi.org/10.1371/journal.ppat.1004050

Yan, Y.-x., Shao, M.-J., Qi, Q., Xu, Y.-S., Yang, X.-Q., Zhu, F.-H., … Zuo, J.-P. (2018). Artemisinin analogue SM934 ameliorates DSS-induced mouse ulcerative colitis via

suppressing neutrophils and macrophages. *Acta Pharmacologica Sinica, 39*(10), 1633–1644. https://doi.org/10.1038/aps.2017.185

Yoshimi, A., Miyazawa, K., & Abe, K. (2016). Cell wall structure and biogenesis in *Aspergillus* species. *Bioscience Biotechnology and Biochemistry, 80*(9), 1700–1711. https://doi.org/10.1080/09168451.2016.1177446

Zheng, J., Li, H., Zhang, X., Jiang, M., Luo, C., Lu, Z., … Shi, J. (2018). Prebiotic mannan-oligosaccharides augment the hypoglycemic effects of metformin in correlation with modulating gut microbiota. *Journal of Agricultural and Food Chemistry, 66*(23), 5821–5831. https://doi.org/10.1021/acs.jafc.8b00829