

A High-Fish Oil Diet Can Significantly Reshape The Gut Microbiota In Mice

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Abstract

Background: Gut microbiota plays an essential role for human health and recent evidence has revealed the beneficial effects of fish oil supplements on the gut microbiota. The present study was to investigate the influence of fish oil on diet-based gut microbiota changes and colitis in mice and whether pyroptosis plays a role in this process.

Results: A high-fish oil diet alleviated colitis, resulted in less weight loss and improved pathological scores. Caspase-1, activated in the dextran sulphate sodium (DSS) group, was suppressed by a high-fish oil diet. AIN-93M significantly decreased the gut microbial diversity of mice, increasing the abundances of *Bacteroides* and *Parabacteroides* and decreasing the abundance of *Odoribacter*. In contrast, gut microbial diversity was maintained in mice fed a high-fish oil diet; the Firmicutes: Bacteroidetes ratio was increased, the abundance of *Parabacteroides* was increased, and that the abundance of *Odoribacter* was decreased.

Conclusion: AIN-93M can decrease gut microbiota diversity, which may be associated with a potential proinflammatory effect. Fish oil has anti-inflammatory effects. It can also restore and maintain microbial diversity and suppress pyroptosis activation.

Background

The gut microbiota plays important roles in metabolism and immunity.¹ This microbial community is shaped from infancy and can be greatly influenced by several factors², among which diet is one of the most important. Many studies have shown that changes in macronutrients can lead to dramatic changes in the gut microbiota.³

Fish oil is regarded as a beneficial food supplement, with documented benefits on cardiac vascular health. The primary active ingredient of fish oil is ω -3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid [EPA, 20:5 (n = 3)] and docosahexaenoic acid [DHA, 22:6 (n-3)]. Studies have also demonstrated the beneficial immunomodulatory effects of ω -3 PUFAs, and the ratio of ω -3 and ω -6 PUFAs was shown to play an important role in inflammation by influencing eicosanoid metabolism.⁴ ω -6 PUFAs are metabolized into several proinflammatory mediators, while ω -3 PUFAs function as competitive inhibitors of enzymes and can be metabolized into several anti-inflammatory mediators.⁴

Few studies have investigated the influence of fish oil on the gut microbiota, and the results are controversial, especially the observed changes in the abundance of different bacteria. Multiple studies have demonstrated that ω -3 PUFAs can increase the abundances of *Lactobacillus* and *Bifidobacteria*,⁵⁻⁹ which are thought to have a beneficial influence on health. Although some studies have shown that ω -3 PUFAs can increase the abundance of Bacteroidetes and decrease the Firmicutes: Bacteroidetes ratio,^{8,10,11} the results of another study supported the opposite conclusion.¹²

Pyroptosis, a form of programmed necrosis, has emerged as a general innate immune effector mechanism. Studies¹³⁻¹⁶ have found that pyroptosis is essential for the development of inflammatory bowel disease (IBD). Two pathways were found to mediate pyroptosis. Pro-caspase-1 is activated by various inflammasome complexes, and Pro-caspase-4/5/11 is also activated by cytosolic lipopolysaccharide. Activated Caspase-1 and Caspase-4/5/11 cleave Gasdermin D, which can form pores on the plasma membrane, inducing pyroptosis. IL-1 β , an inflammatory mediator, was also activated by Caspase-1.¹⁷ Studies¹⁸ found crosstalk between pyroptosis and the gut microbiota, suggesting that pyroptosis plays an essential role in maintaining gut homeostasis. However, few studies have investigated the relationship between fish oil, pyroptosis and the microbiota.

In the present study, we administered dietary interventions to mice to assess the influence of fish oil on the gut microbiota and colitis and its modulation of pyroptosis.

Results

Fish oil alleviates DSS-induced colitis

Mice fed with DSS developed colitis with diarrhea, bloody stool, weight loss and decreased activity from the 3rd day. Death started to occur from the 4th day. Mice from the CD and FD groups showed significant more weight loss than the CC and FC groups. (CD vs CC, $P < 0.05$ day 4-7; FD vs FC, $P < 0.05$ day 5). The CD group showed significantly more weight loss than the FD group. (CD vs FD, $P < 0.05$ day 4-5) (Fig. 1B). There were no deaths in the CC and FC groups, and the death rates of the CD and FD groups were 50% (6/12) and 33.3% (2/6), respectively (Fig. 1A). Colons from animals in the CC and FC groups were significantly longer than those from animals in the CD and FD groups (CC vs CD, $P = 0.133$; FC vs FD, $P = 0.013$). There was no significant difference in colon length between the CD and FD groups ($P = 0.451$) (Fig. 1C). Regarding pathology, the colons from the FC and CC groups had normal mucosal structures with intact crypts. Neither mucosal damage nor infiltration of inflammatory cells was found. For Colon from the DSS groups, colitis of various degrees was observed. In most severe cases, the normal mucosal structure of the anal side disappeared, the crypt structure was completely destroyed, and only some epithelial cells were retained. Inflammation involves the mucosal and submucosal layers, with extensive infiltration of inflammation cells. (Fig. 2) Both the CD and FD groups had significantly higher pathological scores than the CC and FC groups (CD vs CC, $P \leq 0.001$; FD vs FC, $P = 0.008$). Moreover, the pathological score of the FD group was significantly lower than that of the CD group ($P < 0.01$). (Fig. 1D)

Fecal microbiota changes associated with diet.

A comparison of the whole microbiome using Analysis of similarities (ANOSIM) revealed no significant differences between the fish oil and control groups before intervention ($R = 0.1426$, $P = 0.081$, FB vs CB). Both the high-fish oil diet and AIN-93M had a significant influence on the microbiota ($R = 1$, $P = 0.003$, CA vs CB; $R = 0.9185$, $P = 0.001$, FA vs FB). Whereas, here was a significant difference between the fish oil and control groups after intervention ($R = 0.8926$, $P = 0.002$, FA vs CA) (Fig. 3A).

The alpha diversity tests using the Shannon index with the Wilcoxon signed-rank test showed no difference in microbial diversity between the fish oil and control groups before intervention (P=0.1989, FB vs CB). Compared with baseline, the AIN-93M intervention significantly decreased the microbial diversity of the fecal microbiota (P=0.0082, CA vs CB). The high-fish oil diet, on the other hand, had no significant influence on microbial diversity (P=0.7024, FA vs FB) (Fig. 3B).

The MetaStat analysis results showed no significant differences in the abundances of Firmicutes and Bacteroidetes, which were the most abundant phyla (abundance>0.1), in the control group. Compared with baseline, the high-fish oil diet intervention significantly reduced the abundance of Bacteroidetes and increased Firmicutes (Fig. 3C 3E). *Bacteroides*, *Parabacteroides*, *Alistipes* and *Odoribacter* were the most abundant genera (abundance>0.01). AIN-93M significantly increased the abundances of *Bacteroides* and *Parabacteroides* and decreased that of *Odoribacter*. The high-fish oil diet, on the other hand, increased the abundance of *Parabacteroides* and decreased that of *Odoribacter* but had no influence on the abundance of *Bacteroides* (Fig. 3D)

Pyroptosis

Caspase-1, IL-1 β and Gasdermin D expression levels were measured using western blotting. No activation of Caspase-1, IL-1 β or Gasdermin D was found in the CC, FC and FD groups. In the CD group, no activated forms of Caspase-1 and IL-1 β were detected. However, Gasdermin D was activated in the CD group (p=0.0159).

Discussion

A Western-style diet may contribute to the pathogenesis of chronic intestinal diseases, such as inflammatory bowel disease, but the associated mechanisms remain unelucidated. Diet may influence host health by directly affecting nutrient digestion and absorption or by influencing the gut microbiota. The relative deficiency of ω -3 PUFAs in the Western diet has been hypothesized to be one of the potential contributors to its adverse effects.¹⁹ With increasing evidence showing that the gut microbiota has an important impact on human health,¹ it is possible that the disruption of the gut microbiota may promote nutrient deficiency with respect to host health. In this study, we observed that diet plays an essential role in host inflammation and gut microbiota, having a large influence on the structure and diversity of the gut microbial community. By supplementing the diets of mice with ω -3 PUFAs, inflammation was alleviated, the diversity of the microbiota was restored, and its structure was shifted toward an increased Firmicutes: Bacteroidetes ratio.

The results of our study showed that compared to a standard diet, AIN-93M can greatly influence the gut microbiota. AIN-93 was developed by the American Institute of Nutrition Rodent Diets in 1993²⁰. In contrast to the natural diets of mice, the composition of AIN-93 is precise, making it a good tool to study the effects of specific diet compositions and their influence on the gut microbiota. However, the results of the present study showed that AIN-93 significantly influences the gut microbiota of mice by reducing species diversity. Feeding AIN-93M mice can increase the abundance of *Bacteroides*, which was shown to

be significantly more abundant in IBD patients than in healthy controls.²¹ Thus, a better chow with an exact composition and little influence on the normal microbiota of mice is needed for studies on the interaction between specific diet ingredients and the microbiota.

As previously reported, the influence of fish oil on the gut microbiota has been debated and requires further investigation. In our study, mice fed with a high-fish oil diet were observed to have a gut microbiota with greater species diversity, an increased Firmicutes abundance and decreased Bacteroidetes abundance, thereby increasing the Firmicutes: Bacteroidetes ratio. Several studies^{5-9, 22,23} have shown that ω -3 PUFAs can influence specific probiotic bacteria and pathogens, thereby affecting inflammation of the gut. In our study, we showed that ω -3 PUFAs can reverse the increase in the abundance of Bacteroides, which is thought to be a proinflammatory genus. Mice fed with a diet rich in ω -3 PUFAs showed reduced inflammation, indicating that ω -3 PUFAs may function as an anti-inflammatory component by modifying the structure of the gut microbiota.

Pyroptosis plays an important role in innate immunity. Cell experiments²⁴ have found that ω -3 PUFAs suppress pyroptosis by suppressing NLRP3 and activating Caspase-1. In our study, however, we found that Gasdermin D was activated, but not Caspase-1 and IL-1 β . Research²⁵ found that DSS failed to induce colitis in germ-free mice, hinting at the importance of microbiota in the development of colitis. According to the results of our study, DSS may promote pyroptosis by activating Caspase-4/5/11, which are activated by LPS. ω -3 PUFA intervention resulted in an increased Firmicutes: Bacteroidetes ratio and decreased LPS, thus suppressing pyroptosis, which may be a possible mechanism of its anti-inflammatory function.

There are several limitations of this study. The sample size was limited, and additional mice in each group may provide a better understanding of the differences between the groups. Furthermore, we analyzed the fecal microbiota of mice, which can be obtained easily and repeatedly but may not be representative of the microbiota in the host.²⁶ Further study to assess the microbiota directly from the intestine may allow for a better understanding of the interaction between the host and microbiota. Our study focused on the influence of fish oil on the microbiota in mice, and additional studies focusing on the associated metabolic and inflammatory pathways would be of great interest.

Conclusions

In summary, in the present study, we showed that AIN-93M had a large influence on the fecal microbiota of mice, which is believed to be the result of proinflammatory effects. A high-fish oil diet could reverse this effect, maintain a healthy microbiota and alleviate inflammation of the colon. To better understand specific components of diet and its influence on the gut microbiota, a standard diet with defined components and little influence on the gut microbiota is needed for further studies. The results indicated that a high-fish oil diet would be a better choice for further study than AIN-93M. Furthermore, fish oil has the potential to be a prebiotic, maintaining microbial diversity and inhibiting proinflammatory microbiota.

Methods

Study objectives

Thirty male C57BL/6N mice (eight weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. The mice were maintained in the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (CAMS) & Peking Union Medical College (PUMC) under specific pathogen-free (SPF) conditions. The mice were maintained on a 12 h light-12 h dark cycle at $23 \pm 3^\circ\text{C}$, with three mice per cage. The animal protocols were approved by the Peking Union Medical College Hospital Animal Welfare Ethics Committee.

Diets

Two different types of diets were formulated and supplied by Beijing Keao Xieli Feed Co. Ltd. AIN-93M²⁰ is a standardized diet with a precise composition that was developed by the American Institute of Nutrition Rodent Diets in 1993. The high-fish oil diet was customized based on the AIN-93M diet, with 40 g/kg soybean oil being replaced by 30 g/kg soybean oil and 10 g/kg fish oil. The detailed compositions of the diets are shown in Table 1.

Table 1
Detailed compositions of the intervention diets

Material(g/kg)	AIN-93M	Customized diet
Casein	140.00	140.00
L-Cystine	1.80	1.80
Corn Starch	465.69	465.69
Maltodextrin	155.00	155.00
Sucrose	100.00	100.00
Cellulose	50.00	50.00
Soybean Oil	40.00	30.00
Fish Oil*	0.00	10.00
Choline Bitartrate	2.50	2.50
AIN Mineral Mix	35.00	35.00
AIN Vitamin Mix	10.00	10.00
BHT	0.01	0.01

*Fish oil was obtained from Chengdu Aowei Health Tech Co. Ltd. Every 100 g of fish oil contained 18.2 g of EPA and 10.3 g of DHA.

Experimental design

Male C57BL/6N mice were randomly assigned to two groups using a computer based random order generator: the control (C, n = 18) and fish oil (F, n = 12) groups. All mice were fed with regular diets (provided by Beijing Vital River Laboratory Animal Technology Co., Ltd.) and purified water after birth until the dietary intervention. The control group was fed the AIN-93M diet and purified water, and the fish oil group was fed the customized diet and purified water. The dietary intervention duration was 3 weeks. Feces were collected from each mouse (except 6 from the DSS control group to achieve equal sample size for microbiota analysis) before and at the end of the intervention (Fig. 5). Feces were collected immediately after they were produced and stored at -80°C.

After two weeks of dietary intervention, the mice were then randomly assigned into four subgroups using the same random order generator: control group without DSS (CC n = 6), control group with DSS (CD n = 12), fish oil group without DSS (FC n = 6) and fish oil group with DSS (FD n = 6). For groups with DSS, 2.5% dextran sodium sulfate (DSS, supplied from MP Biomedicals, LLC) was added to purified water for 5 days to induce acute colitis. The weight of each mouse was measured every day. Seven days after DSS initiation, the mice were anesthetized with 4% chloral hydrate (10 μ L/g, through intraperitoneal injection) and sacrificed by breaking the neck. The whole colon was collected for further study.

Due to different appearance of feed and water, the experiment could neither be blinded to whether the animal was fed AIN-93M or customized diet, nor to whether the animal was fed purified water or purified water dissolved with DSS.

Mouse colon inflammation assessment

The length of the whole colon of each mouse was measured. The colon was then cut in half along the long axis. Half of the sample was taken to the Peking Union Medical College Hospital Pathology Department for specimen preparation and HE staining.²⁷ The pathological score²⁸ was calculated by a pathological specialist who was not a member of our research group and was blinded to the intervention of mice.

Measurement of pyroptosis activation in colonic tissue

The other half of the colon was washed with cold phosphate-buffered saline and stored at -80°C until use. Colonic tissue was homogenized using RIPA lysis buffer (Applygen Technologies Inc.). Tissue homogenates were transferred to microcentrifuge tubes, vortexed, and centrifuged at 4°C for 10 min at 12,000 \times g. Two microliters of supernatant from each sample was withdrawn to assess the protein concentration using a Coomassie brilliant blue kit (Applygen Technologies Inc.) according to the manufacturers' protocol. Protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The activation of Caspase-1, IL-1 β and Gasdermin D was assessed using antibodies from Abcam (ab179515, ab9722 and ab209845).

Microbiota diversity analysis

Total genomic DNA was extracted from each fecal sample using the sodium dodecyl sulfate (SDS) method and 1% agarose gels. The V4 region of the 16S rRNA gene was PCR amplified using the 515F

(GTGCCAGCMGCCGCGGTAA) and 806R (GGA CTACHVGGGTWTCTAAT) primers with 15 μ L of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μ M primers and 10 ng of template DNA. The thermal cycling conditions were an initial denaturation step at 98°C for 1 min; 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s and elongation at 72°C for 30 s; and finally 72°C for 5 min. Finally, sequencing libraries were generated using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations, and index codes were added. The library quality was assessed using a Qubit® 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system, and then, the samples were sequenced on an Illumina NovaSeq platform to generate 250 bp paired-end reads.

Bioinformatics analysis

Paired-end reads were merged using FLASH (V 1.2.7). The data were then filtered to obtain clean, high-quality tags according to the QIIME (V 1.9.1) quality-control process. The tags were compared with the reference database (Silva Database) using the UCHIME algorithm to remove chimeric sequences. Operational taxonomic unit (OTU) grouping was performed using Uparse (V 7.0.1001). Sequences with \geq 97% similarity were assigned to the same OTUs. The Silva Database based on the Mothur algorithm was used to annotate taxonomic information. Alpha diversity was calculated with QIIME (V 1.7.0) and displayed with the R software environment (V 2.15.3). Beta diversity was calculated using QIIME (V 1.9.1), and principal component analysis (PCA) was performed using the FactoMineR and ggplot2 packages in the R software environment (V 2.15.3).

Abbreviations

DSS: dextran sulphate sodium

PUFAs: Polyunsaturated fatty acids

EPA: eicosapentaenoic acid

DHA: docosahexaenoic acid

IBD: inflammatory bowel disease

IBS: irritable bowel syndrome

UC: ulcerative colitis

CAMS: Chinese Academy of Medical Science

PUMC: Peking Union Medical College

SPF: specific pathogen free

SDS: sodium dodecyl sulfate

OTU: operational taxonomic unit

LEfSe: LDA effect size

ANOSIM: Analysis of similarities

Declarations

Ethics approval and consent to participate

Animal protocols were approved by the Peking Union Medical College Hospital Animal Welfare Ethics Committee. all methods were performed in accordance with the Animal management regulations and guidelines from the ethics committee. The study was carried out in compliance with the ARRIVE guidelines

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the repository of Novogene Co. Ltd. And the project number is P101SC18051695-02-F002-B1-41.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

HS performed the animal experiment and was one of the major contributors in writing the manuscript. DC and BT helped modified the manuscript. All authors read and approved the final manuscript.

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Figures

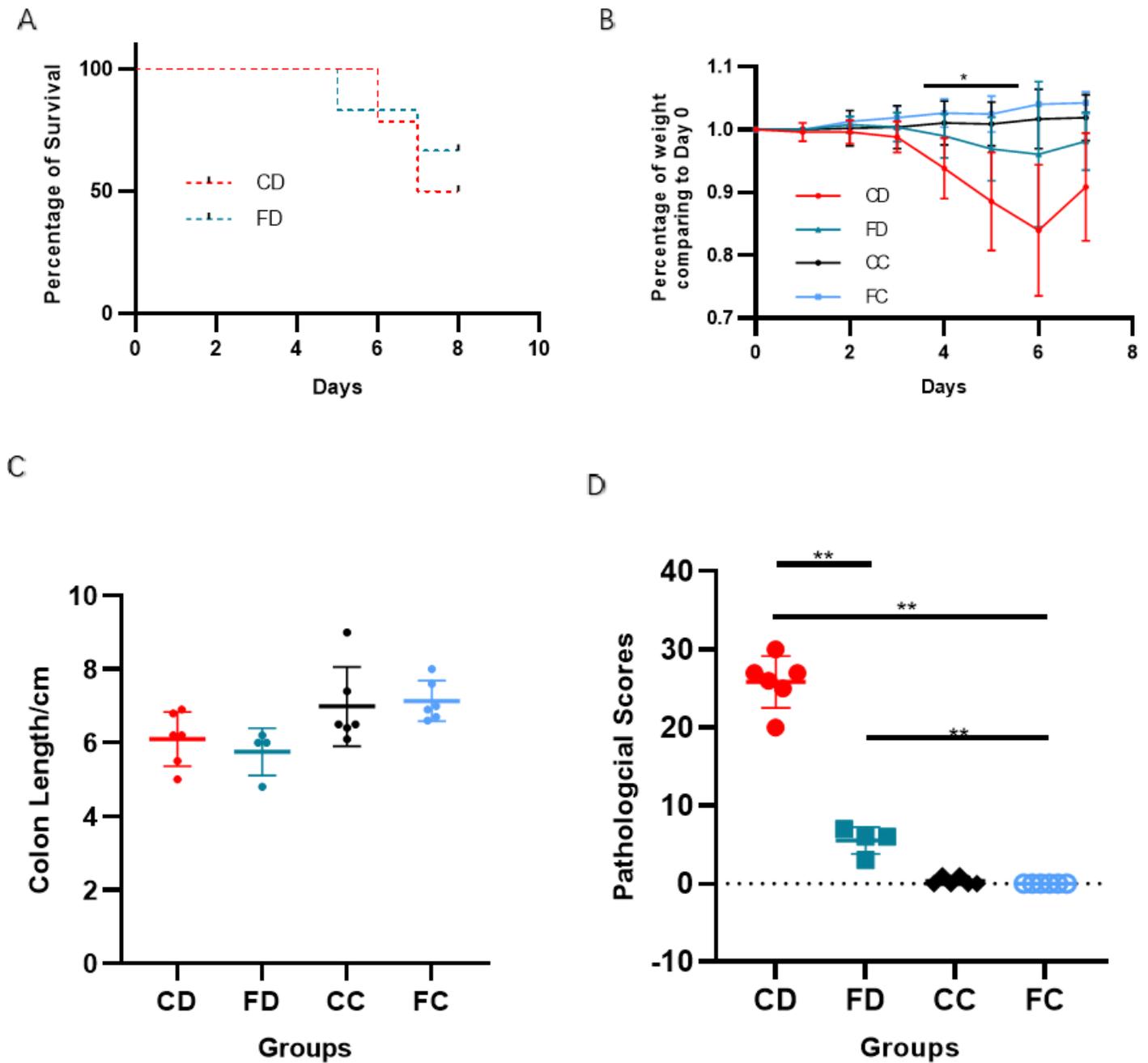


Figure 1

Influence of AIN-93M and high-fish oil diet on colitis in mice. (A) Survivorship curve. (B) Weight change. The weight of the CD group was significantly lower than that of the FD group on days 4 and 5 ($P < 0.05$). (C) Colon length. There were no significant differences between the CD and FD groups ($P = 0.451$). (D) Pathological score. The pathological score of FD was significantly lower than that of CD ($P < 0.01$). Data shown in mean \pm SD. * $P < 0.05$; ** $P < 0.01$. The CD group was fed AIN-93M and DSS to induce colitis. CC group were fed with AIN-93M but no DSS. FD group were fed with high-fish oil diet and DSS. The FC group was fed a high-fish oil diet but no DSS.

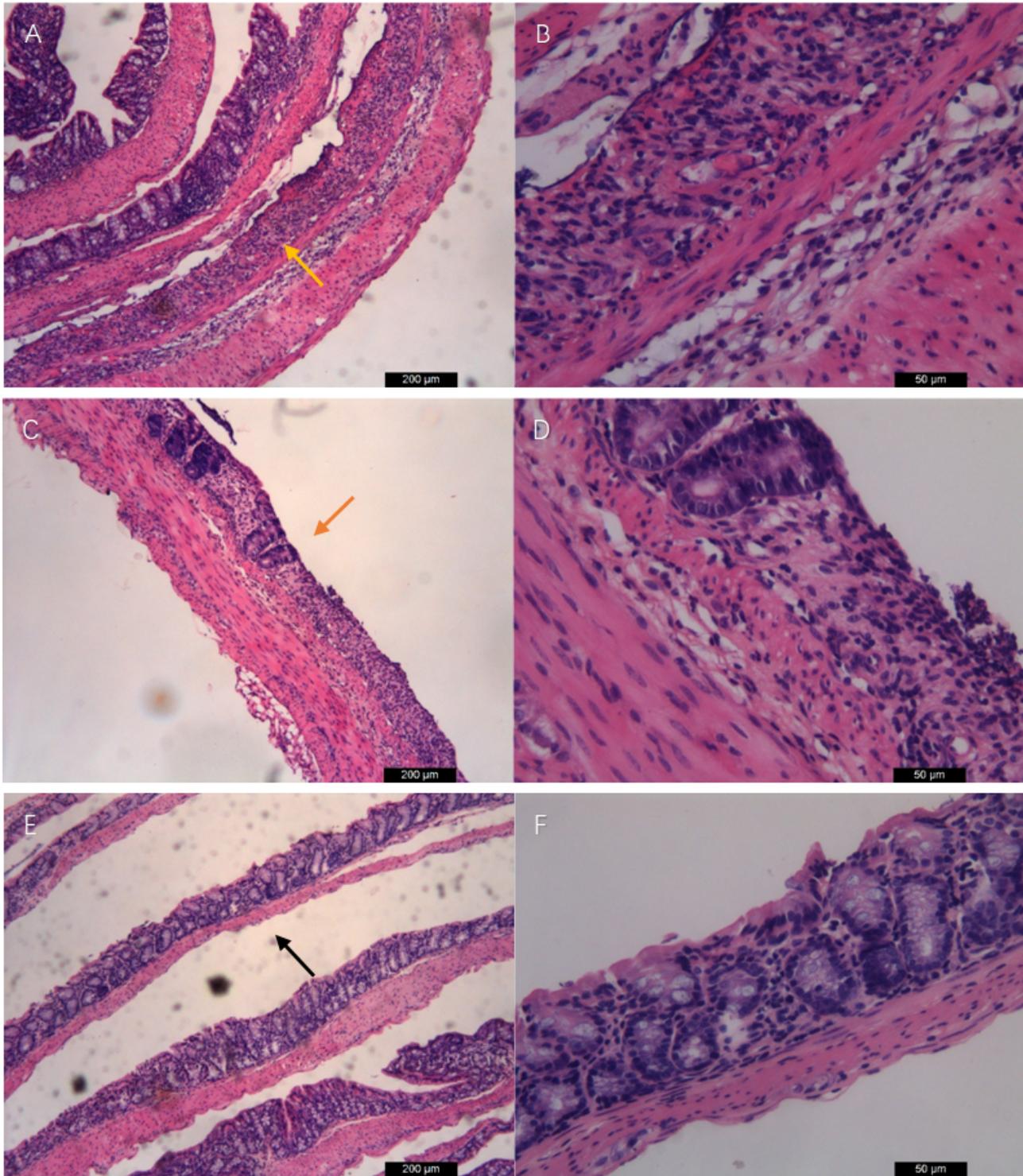


Figure 2

Pathological features of the different groups. The colons from the FC and CC groups had normal mucosal structures with intact crypts. Neither mucosal damage nor infiltration of inflammatory cells was found. The colons from the CD and FD groups showed colitis of various degrees. In the most severe cases, the normal mucosal structure disappeared on the anal side, the crypt structure was completely destroyed, and only some epithelial cells were retained. Inflammation involves the mucosal and

submucosal layers, with a large number of inflammatory cells found. (A) CD group (5×, H&E staining); (B) amplification of yellow arrow in picture A (20×); (C) FD group (5×, H&E staining); (D) amplification of orange arrow in picture C (20×); (E) CC group (5×, H&E staining); and (F) amplification of black arrow in picture E (20×),

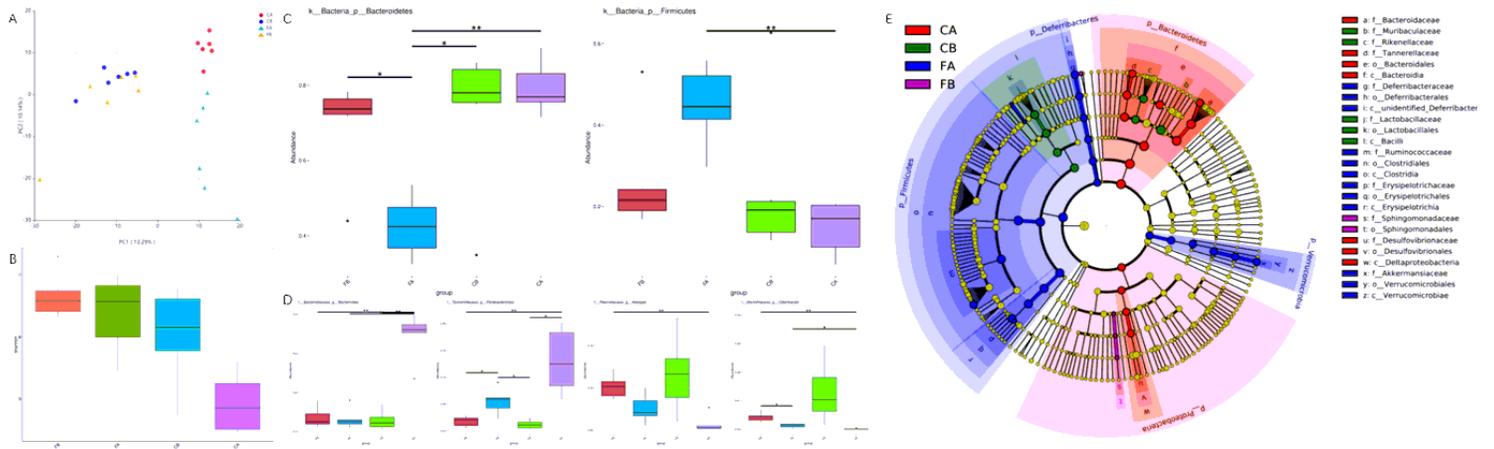


Figure 3

Influence of AIN-93M and high-fish oil diet on the gut microbiota of mice. (A) Principal component analysis. Both the AIN-93M and high-fish oil diets altered the gut microbiota, but in different directions. (B) Shannon index with Wilcoxon signed-rank test. The CA group had a significantly lower Shannon index value than the other 3 groups, which had similar index values. (C) MetaStat analysis at the phylum level. The FA group had a higher abundance of Firmicutes and a lower abundance of Bacteroidetes compared to the other 3 groups, which had similar abundances. (D) MetaStat analysis at the genus level. The CA group had a significantly higher abundance of Bacteroides than the other three groups. (E) Cladogram of LDA effect size (LEfSe) analysis. Firmicutes played a dominant role after the high-fish oil diet intervention, while Bacteroidetes and Proteobacteria played a dominant role after AIN-93M intervention.

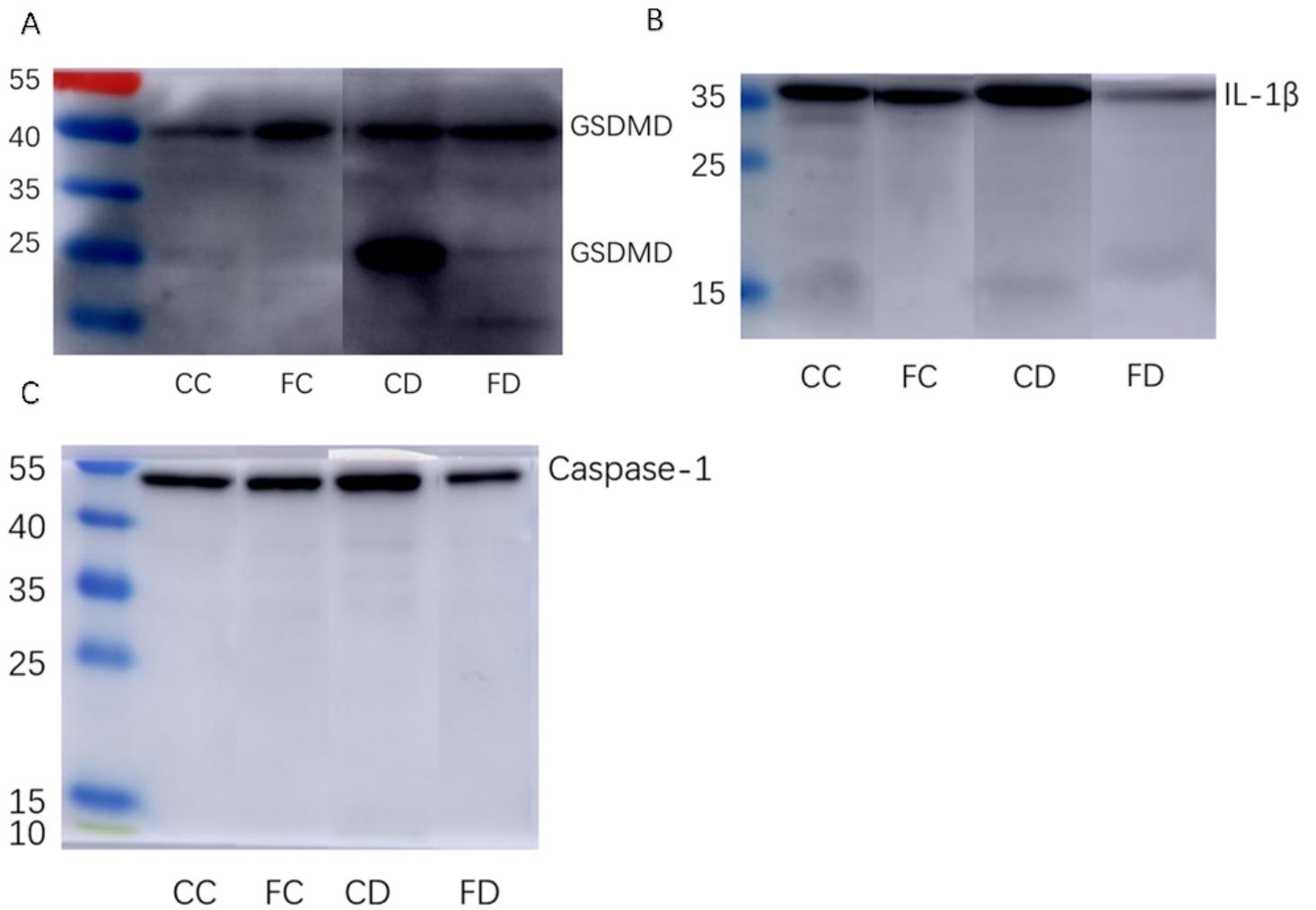


Figure 4

Detection of Caspase-1, IL-1 β and Gasdermin D. (A) The activation of Gasdermin D was found only in the CD group. (B) No activated form of IL-1 β was found in any group. (C) No activated form of Caspase-1 was found in any group.

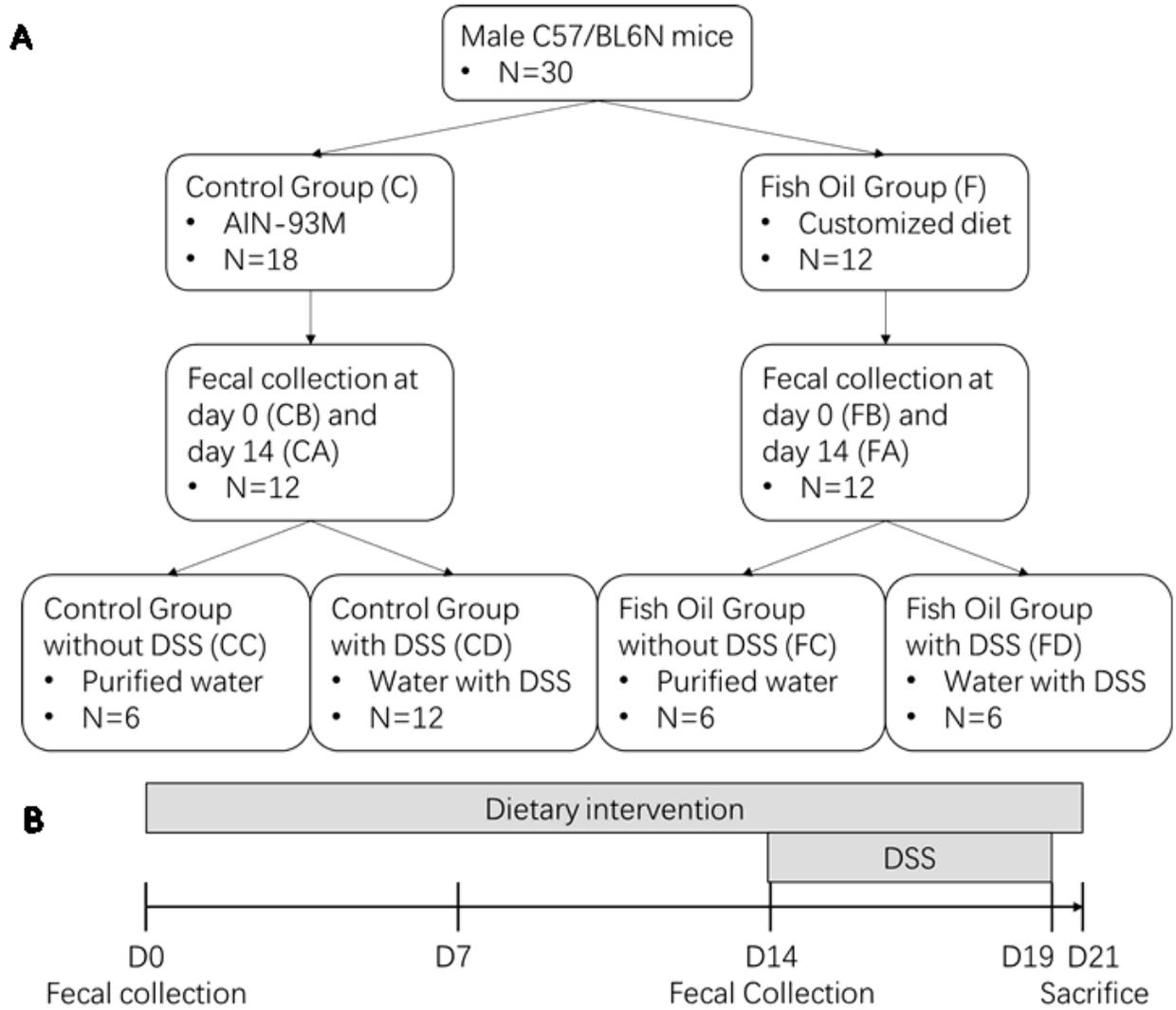


Figure 5

Flow chart of the experiment. A total of 30 male C57/BL6N mice were randomly divided into a control group (N=18) fed AIN-93M and a fish oil group (N=12) fed a high-fish oil diet. Feces were collected from each mouse on day 0 and day 14 except for those in the CD group, in which 6 out of 12 mice were randomly selected for fecal collection for statistical analysis reasons. The mice were then subdivided into CC, CD, FC and FD groups. For the CD and FD groups, DSS was administered for 5 days to induce colitis. On day 21, the mice were sacrificed for further analysis.