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Fungal-bacterial interactions in mice with dextran sulfate sodium (DSS)-induced acute and chronic colitis

Xinyun Qiu 1,2#, Xia Li 1, Zhe Wu 1, Feng Zhang 1, Ning Wang 1, Na Wu 3, Xi Yang 4, Yulan Liu 1*.

1Department of Gastroenterology, Peking University People's Hospital, Beijing, China.
2Department of Gastroenterology, First Affiliated Hospital of Nanjing Medical University, Nanjing, China.
3Institute of Clinical Molecular Biology & Central Laboratory, Peking University People's Hospital, Beijing, China.
4CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China.

# Dr. Xinyun Qiu is a doctoral candidate in Department of Gastroenterology, Peking University People's Hospital from September 2012 to July 2015, and now he is a resident physician in Department of Gastroenterology, First Affiliated Hospital of Nanjing Medical University.

*Corresponding author. Mailing address for Yulan Liu:
Department of Gastroenterology, Peking University People's Hospital
No. 11 Xizhimen South Street, Xicheng District, Beijing 100044, China.

Phone: (8610)8832-5559; Fax: (8610)6831-8386; E-mail: liuyulan@pkuph.edu.cn
ABSTRACT

The commensal intestinal microbiota plays critical roles in the initiation and development of inflammatory bowel diseases (IBD). However, the importance of intestinal fungi and their interactions with bacteria in the pathogenesis of IBD is unclear. In this study, anti-fungal drugs (fluconazole or Amphotericin B [AmpB]) were administered to control mice and those treated, acutely (A-DSS) and chronically (C-DSS) with DSS. The severity of colonic inflammation was assessed, and pro-inflammatory cytokine levels in the colonic mucosa and serum were detected by real-time PCR and multiplex ELISA assay, respectively. The colonic bacterial 16S rDNA V3 region was analysed in normal and anti-fungal drug-treated mice by pyrosequencing. Specific bacterial genera related to IBD, butyryl-CoA/acetate-CoA transferase (a key transferase for butyrate production in bacteria) and tight-junction proteins (occludin and ZO-1) in the colonic mucosa were detected by real-time PCR and/or western blot. The results showed that compared with controls, intestinal Bacteroides, Alistipes, and Lactobacillus were increased in fluconazole treated mice; while only Alistipes was increased in AmpB treated mice. Clostridium cluster XIVa and butyryl-CoA/acetate-CoA transferase levels were reduced in both groups. Treatment with a high dose of anti-fungal drugs dampened colonic occludin and ZO-1, aggravated A-DSS colitis, and exhibited no beneficial role in C-DSS colitis. However, reducing fluconazole concentration to an appropriate dose attenuated C-DSS colitis. In conclusion, fungi are important co-operators with bacteria in maintaining intestinal micro-ecological equilibrium. Extensive...
clearance of gut fungi could disrupt intestinal bacterial homeostasis and have an adverse impact on IBD. Studies investigating the intestinal fungal-bacterial interactions would aid the appropriate use of anti-fungal drugs in treating IBD.

**Key words:** Inflammatory bowel disease, Colitis, Intestinal microbiota, Fungi, Fungal-bacterial interactions.

**BACKGROUND**

Inflammatory bowel diseases (IBDs) such as ulcerative colitis (UC) and Crohn’s disease (CD), are caused by multiple factors, including genetics, environment, immune dysfunction, and disturbance of the intestinal microbiota. The latter comprises a large and diverse community of bacteria, fungi, viruses, and archaea. The intestinal microbiota plays vital roles in developing and maintaining host immunology, physiology, nutrition, and metabolism.

Patients with IBD are usually characterised by disturbances in their intestinal microflora. Several studies have reported an increased abundance of *Gammaproteobacteria* and *Enterobacteriaceae*, and decreased abundance of *Firmicutes* in both UC and CD patients. Numbers of bacteria producing butyrate, an important short chain fatty acid (SCFA) that plays a major role in promoting visceral function and integrity, are significantly decreased in the gut of IBD patients. Additionally, pathogenic sulfur-reducing, bile-tolerant bacteria, which produce toxic and proinflammatory hydrogen sulfide and exacerbate intestinal inflammation, are increased in the gut of UC patients. Besides bacteria, intestinal fungi also exhibit a significant change during inflammatory condition. Denaturing gradient gel electrophoresis analysis of fungal 18S rDNA showed different fungal profiles between UC patients and healthy individuals, and an increased proportion of *Candida albicans* was found in the colons of CD patients, causing aggravated mucosal injury and generation of anti-*Saccharomyces cerevisiae* antibodies (ASCA).

Pathogenic gut microbiota could be an important inducer in IBD initiation and perpetuation, and
past studies have verified that elimination of gut bacteria by broad-spectrum antibiotics is effective in inducing IBD remission\textsuperscript{15}. However, to our knowledge, the role of anti-fungal drugs in treating IBD has not been demonstrated conclusively. Fungi and bacteria coexist in the gastro-intestinal (GI) canal, where they interact extensively with each other\textsuperscript{16, 17}. Fungi usually bloom during antibiotic perturbations, especially in immunocompromised patients such as those with acquired immune deficiency syndrome (AIDS), some cancers, and transplant recipients\textsuperscript{18}. Dollive et al\textsuperscript{18} demonstrated an outgrowth of fungi in both short-term and long-term antibiotic treatment in mouse models and found that \textit{Candida} can persist in the gut at a high level for eight weeks after the cessation of antibiotics. However, whether anti-fungal drugs have an effect on intestinal bacteria is unknown.

The objective of this study was to elucidate the interactions between intestinal bacteria and fungi, and to investigate the role of intestinal fungi in IBD. To this end, we investigated the effects of anti-fungal drugs (fluconazole and amphotericin B [AmpB]) in dextran sulphate sodium (DSS)-induced murine acute and chronic IBD models, and compared the bacterial composition in the colonic mucosa between normal and anti-fungal drug-treated mice. Our results unravel the interaction between bacteria and fungi in the gut and further our knowledge of microbial homeostasis in the GI canal.

\textbf{MATERIALS AND METHODS}

\textbf{Animal protocols}

Six-week-old C57B/L6J mice were purchased from the Experimental Animal Center, Academy of Military Medical Sciences (Beijing, China), housed four to five per cage and placed on standardized rodent food and distilled water for four weeks prior to the study to stabilize their microflora. To induce acute DSS-colitis, mice were given drinking water containing 2.5\% (w/v) DSS (MP Biomedicals, Aurora, OH) ad libitum for seven days and distilled water for two additional days before sacrifice. To induce chronic DSS-colitis, mice were given four cycles of 7-day 2.5\% (w/v) DSS followed by 7-day
Effect of anti-fungal drugs in mice with acute-DSS (A-DSS) colitis

To induce acute DSS-colitis (A-DSS), mice were given water containing 2.5% (w/v) DSS (molecular weight 36–50 kDa; MP Biomedicals) *ad libitum* for seven days followed by water without DSS for two additional days before sacrifice. Two anti-fungal drugs (fluconazole and AmpB) were tested in the A-DSS model in separate experiments. In the first experiment, animals were divided into the following four treatment groups (n = 8–10 per group): normal, A-DSS, fluconazole, and A-DSS + fluconazole. In the second experiment, the same groups were used, but fluconazole was replaced with AmpB. To deplete intestinal fungi using fluconazole, the fluconazole and A-DSS + fluconazole groups were exposed to fluconazole (0.5 mg/mL, Sigma-Aldrich, St Louis, MO) via their drinking water on days 1–23. In the A-DSS and A-DSS + fluconazole groups, 2.5% (w/v) DSS was added to the drinking water only on days 15–21 (Table 1). To deplete intestinal fungi using AmpB, the AmpB and A-DSS + AmpB groups were administered AmpB (an insoluble drug, 1 mg/kg bodyweight) intragastrically on days 1–23 using the method reported by Reikvam et al. The normal and A-DSS mice were administrated distilled water intragastrically as a control. In the A-DSS and A-DSS + AmpB groups, 2.5% (w/v) DSS was added to drinking water only on days 15–21 (Table 1). Mice in all groups were weighed on days 14–23. The mice were sacrificed on day 23, and the colon tissues (3 cm above the anal canal) were extracted and stained with haematoxylin and eosin as described previously. Inflammation was graded by two independent blinded observers according to an established grading system.

Effect of different concentrations of fluconazole in mice with chronic-DSS (C-DSS) colitis
To induce C-DSS colitis, mice were treated with four 14-day cycles consisting of seven days of water containing 2.5% DSS followed by seven days of distilled water (DSS + water) before being sacrificed, according to the protocol described by Batra et al. with modifications. Mice were randomly divided into four groups: C-DSS, C-DSS + Flu1, C-DSS + Flu2, and C-DSS + Flu3. Three different concentrations of fluconazole (Flu1: 0.5 mg/mL [fungi-depleted concentration], Flu2: 0.125 mg/mL, and Flu3: 0.0625 mg/mL) were added to the water on the fourth DSS + water cycle (Figure S1). Mice in all groups were weighed on the fourth DSS + water cycle (day 43–56), colon tissue was collected, and the inflammation was graded as stated earlier.

**DNA isolation**

Mouse colons were opened longitudinally and briefly washed in cold sterile phosphate-buffered saline (PBS) three times immediately after sacrifice. The colonic mucosal samples (2 cm above the anal canal) were collected, frozen in liquid nitrogen, and stored at −80 °C until further processing. DNA was extracted from the colonic tissue samples (referred to as mucosa in our study) by using a FastDNA SPIN Kit for Feces (MP Biomedicals) according to the manufacturer’s instructions and was quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific).

**Library construction**

The 16S rRNA gene V3 region (representing bacteria) in the colonic mucosal samples of mice was amplified and pyrosequenced. We amplified 16S rDNA with the universal primers 341F (5′-NNNNNNNCCTACGGGAGGCAGCAG-3′) and 534R (5′-NNNNNNNNATTACCGCGGCTGCTGAG-3′). The primer sets were modified with Illumina adapter regions for sequencing on the Illumina GAIIx platform, and the reverse primers were modified with an 8-bp Hamming error-correcting barcode to distinguish the samples. The mucosal DNA template (200 ng) was combined with 0.25 µL HotStarTaq Plus DNA Polymerase (Qiagen), 1 µL
dNTPs, 5 µL polymerase chain reaction (PCR) buffer, and 2.5 pmol of each primer in a total volume of 50 µL. The PCR cycle consisted of an initial step at 95 °C for 5 min; 25 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 60 s; and a final extension at 72 °C for 10 min. PCR products were verified by 1.5% (w/v) agarose gel electrophoresis, stained with ethidium bromide, photographed under UV illumination, and purified with the QIAquick® gel extraction kit (Qiagen).

Real-time PCR

To detect the levels of inflammatory cytokines (IL-6, IL-17A, and IFN-γ) and tight-junction proteins (occludin and ZO-1) in the colonic mucosa, total RNA was extracted from the proximal and distal colon by the TRIzol® method (Gibco) and converted into cDNA. Rpl32 was used as the internal control as it is stable during intestinal inflammation. The primers used are listed in Table 2. To detect 16S rDNA (representing bacteria), 18S rDNA (representing fungi), and several specific bacterial genera in the colonic mucosa, β-actin was used as the internal control. PCR reactions consisting of 100 ng template cDNA (or DNA), 0.8 µL (10 nM) each primer, 10 µL SYBR® Green PCR master mix (TOYOBO), and 7.4 µL of water in a total volume of 20 µL were performed on an ABI StepOne Plus Sequence Detection System (Applied Biosystems); the thermocycling reactions involved the following steps: 95 °C for 1 min; 40 cycles of 95 °C for 15 s, 56 °C for 15 s, and then 72 °C for 45 s. The primers used are listed in Table 3. All samples were analysed in duplicate in a single 96-well reaction plate, and the data were analysed according to the 2^−ΔCT method.

Pro-inflammatory cytokines in the serum

Blood was collected, coagulated, centrifuged (10 min at 3000 × g), and stored at −80 °C until further processing. Inflammatory cytokine (IL-6, IL-17A, and IFN-γ) concentrations in the serum were determined using Milliplex™ MAP mouse immunoassay kits (Millipore) according to the manufacturer’s instructions.
Bioinformatics analysis

Sequences of the V3 region of 16S rDNA in mouse colonic mucosa were detected using an Illumina HiSeq™ 2000 platform (reconstructed cDNA sequence: 2 × 150 bp). Ribosomal Database Project (RDP) Classifier 2.8 was used for taxonomical assignment of all the sequences at 50% confidence after the raw sequences were identified by their unique barcodes. Bacterial genera with an average relative abundance of ≥0.01 in colonic samples were identified as major genera. Operational taxonomic units (OTUs) present in ≥50% of the mucosal specimens were deemed core OTUs. Partial least-squares discriminant analysis (PLS-DA) of core OTUs was performed using Simca-P version 12 (Umetrics) to visualise and cluster the fungal community into different groups.

Bacterial genera and butyryl-CoA/acetate-CoA transferase concentration in colonic mucosa

We utilised previously reported broad-spectrum primers to amplify the targeted genomic DNA of Bacteroides$^{25}$, Alistipes$^{26}$, Lactobacillus$^{27}$, Clostridium cluster XIVa$^{25}$, Lachnospiraceae incertae sedis$^{28}$, Helicobacter$^{29}$, and the butyryl-CoA/acetate-CoA transferase gene$^{25}$ in order to detect and quantify the different bacterial genera and the level of butyrate-producing bacteria in the colonic mucosa of mice by real-time PCR, with β-actin as the internal control.

Western blot analysis.

Proximal and distal colonic tissues from normal, fluconazole-, and AmpB-treated mice were disrupted by homogenization on ice and centrifuged at 4 °C (12,000 × g, 15 min). The supernatants were collected, and protein concentrations were determined by the Bradford assay with bovine serum albumin. Equal amounts of protein (70 ng/lane) were separated on 8% SDS-PAGE, transferred to a nitrocellulose membrane, blocked in 5% skim milk, and incubated with antibodies against Occludin
(1:200; Santa Cruz Biotechnology, Santa Cruz, CA), ZO-1 (1:100; Santa Cruz Biotechnology), and β-actin (1:2000; Cell Signaling) at 4 °C overnight. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:400; Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h at room temperature. After one more wash, immunoreactive bands were visualised with ECL detection reagents (Thermo Scientific).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Statistical Package for Social Sciences version 17.0 (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) and Mann–Whitney U-tests were used for analysis. Differences were considered significant at a $P$ value of less than 0.05.

RESULTS

Intestinal fungi-depletion by fluconazole aggravates A-DSS colitis in mice

The A-DSS + fluconazole group exhibited the most severe colonic inflammation, greatest weight loss (Figure 1A), shortest colon length (Figure 1B), and highest histological score (Figure 1C) among the four groups (normal, fluconazole, A-DSS, and A-DSS + fluconazole), followed by the A-DSS group. The A-DSS + fluconazole and A-DSS mice showed higher mRNA levels of pro-inflammatory cytokines (IL-6, IL-17A, and IFN-γ) in the colonic mucosa and increased serum levels of pro-inflammatory cytokines compared with the normal and AF groups (Figure 2A-F). Notably, the A-DSS + fluconazole group exhibited the highest pro-inflammatory cytokine level in both the colonic mucosa and peripheral blood, followed by the A-DSS group (Figure 2A-F). No obvious inflammation was detected in the normal and fluconazole-treated mice (Figures 1 and 2).
Intestinal fungidepletion by AmpB also aggravates A-DSS colitis in mice

The A-DSS + AmpB group exhibited the most severe colonic inflammation among the normal, AmpB, A-DSS, and A-DSS + AmpB groups. Both the A-DSS and A-DSS + AmpB mice showed significant weight loss compared with the normal and AmpB mice (Figure 3A). Moreover, the weight lost by the A-DSS + AmpB mice was markedly higher than that lost by the A-DSS group on days 19 and 22. (Figure 3A) The A-DSS + AmpB group showed the shortest colon length (Figure 3B) and highest histological score (Figure 3C) among the four groups, followed by the A-DSS group; the A-DSS + AmpB and A-DSS mice revealed higher mRNA levels of pro-inflammatory cytokines (IL-6, IL-17A, and IFN-γ) in their colonic mucosa compared with the normal and AmpB groups (Figure S2). Notably, the A-DSS + AmpB group showed the highest pro-inflammatory cytokine concentration in the colon, followed by the A-DSS group (Figures 3 and S2). No obvious inflammation was detected in the normal and AmpB-treated mice (Figure 3 and S2).

Comparison of 16S rDNA and 18s rDNA levels in the colonic mucosa of normal and anti-fungal drug-treated mice

18S rDNA and 16S rDNA primer-amplified fragments were used to quantitatively analyse the fungal and bacterial content of the gut, respectively. Anti-fungal drugs (fluconazole and AmpB) significantly decreased the 18S rDNA levels in the colonic mucosa. Interestingly, the 16S rDNA levels were not significantly different between the anti-fungal drug-treated and normal groups (Figure S3 and S4).

Bacterial compositions in the normal and anti-fungal drug-treated mice

To observe the effects of the anti-fungal drugs (at a dosage that depleted intestinal fungi) on the intestinal microbiota and to compare the intestinal bacterial communities between normal and anti-fungal drug-treated mice, we investigated the bacterial composition of the colonic mucosa from
normal and fluconazole-treated mice by high-throughput sequencing. Overall, 91,824,505 high-quality reads of the 16S rDNA V3 region (~5,739,031 reads/sample) were obtained for taxonomic analysis. The rarefaction curve shows the observed number of OTUs on sequence counts at different sequencing depths (Figure S5); the rarefaction curve was saturated, indicating that no new OTUs were detected when the sequencing depth was increased. A total of 340 OTUs (>10 reads for each OTU) were identified in the colonic mucosa of both groups (Figure 4A), 268 overlapping OTUs were detected between the two groups, while 20 and 52 OTUs were uniquely present in the normal and fluconazole mice, respectively, indicating a greater bacterial diversity in the gut of fluconazole-treated mice. PLS-DA (Figure 4B) based on the core OTUs revealed that the colonic mucosal bacteria in the normal and fluconazole mice could be classified into two different clusters. Three dominant phyla (Firmicutes, Proteobacteria, and Bacteroidetes) accounted for more than 97% in all of the samples (Figure 4C). Compared with the normal control group, the prevalence of Firmicutes and Bacteroidetes was much higher, while that of Proteobacteria was much lower, in the fluconazole-treated mice (Figure 4C). In addition, eight major genera (Helicobacter, Clostridium cluster XIVa, Anaerostipes, Lachnospiracea incertae sedis, Lactobacillus, Mucispirillum, Marvinbryantia, and Alistipes) were detected in the colonic mucosa of normal mice (Figure 4A), while thirteen major genera (Helicobacter, Lactobacillus, Clostridium cluster XIVa, Lachnospiracea incertae sedis, Alistipes, Marvinbryantia, Bacteroides, Hallella, Xylanibacter, Barnesiella, Anaerostipes, Flavonifractor, and Prevotella) were detected in the fluconazole group (Figure 4B).

We further detected the seven bacterial genera (Bacteroides, Alistipes, Lactobacillus, Clostridium cluster XIVa, Lachnospiracea incertae sedis, and Helicobacter) that are associated with intestinal inflammation and carcinoma, as well as the butyryl-CoA/acetate-CoA transferase (a key transferase in butyrate production in bacteria) gene level in the colonic mucosa of mice, and found that Bacteroides, Alistipes, and Lactobacillus were significantly higher in the colonic mucosa of fluconazole-treated mice (Figure 5A-C), while Clostridium cluster XIVa and butyryl-CoA/acetate-CoA transferase levels were significantly lower, than in the normal control mice (Figure 5D and 5F). The levels of Lachnospiracea incertae sedis and Helicobacter, two abundant bacteria in the mouse gut,
were not markedly different between the fluconazole-treated and normal mice (Figure 5E and 5G). To study the effects of AmpB on the intestinal microbiota, we compared the differences in the levels of *Bacteroides*, *Alistipes*, *Lactobacillus*, *Clostridium* cluster XIVa, *Helicobacter*, and *butyryl-CoA/acetyl-CoA transferase* in the colonic mucosa of normal and AmpB-treated mice by real-time PCR. Interestingly, the level of *Alistipes* increased, while that of *Clostridium* cluster XIVa and *butyryl-CoA/acetyl-CoA transferase* decreased in the colonic mucosa of the AmpB-treated mice compared with the normal control (Figures S6B, D and F); the other four genera did not show remarkable differences between the two groups (Figure S6A, C and E).

**Occludin and ZO-1 levels decreased in the colonic mucosa of anti-fungal drug-treated mice**

The levels of two tight-junction proteins (occludin and ZO-1) were examined in the colon tissue of normal and anti-fungal drug (fluconazole and AmpB)-treated mice. The expression levels of occludin and ZO-1 were significantly lower in the colon tissue of anti-fungal drug (fluconazole and AmpB)-treated mice than of the normal mice, as analysed by real-time PCR (Figure 6A-B) and western blot (Figure 6C-D), respectively.

**Effects of fluconazole treatment in mice with C-DSS colitis**

To observe the effects of fluconazole treatment on C-DSS murine colitis, we added fluconazole in the drinking water at the fourth DSS + water cycle at three different concentrations. Among the four groups (C-DSS, C-DSS + Flu1, C-DSS + Flu2, and C-DSS + Flu3 groups) of mice, the C-DSS and C-DSS + Flu1 (fungi-depleted concentration of fluconazole) groups showed the greatest weight loss (Figure 7A), shortest colon length (Figure 7B), and highest pathological score (Figure 7C) and pro-inflammatory cytokine level in the colonic tissue and serum (Figure 8), followed by the C-DSS + Flu3 and C-DSS + Flu2 groups (Figures 7 and 8). However, no difference was detected in the degree of inflammation between the C-DSS and C-DSS + Flu1 mice (Figures 7 and 8).
DISCUSSION

Intestinal fungi and bacteria are important micro-ecological components that play crucial roles in maintaining the health and functioning of the GI canal. Imbalance in the composition of both microbiota has been implicated in the development of IBD\textsuperscript{17,31}. Thus, modulation of gut microbiota in IBD patients is a growing area of scientific research. Several studies have suggested that IBD patients exhibit increased numbers of mucosal-associated bacteria\textsuperscript{32}, and gut inflammation could develop from bacterial invasion in the intestinal mucosa and failed clearance of the intruders by the host immune system\textsuperscript{33}. Therefore, antibiotics were proposed as a beneficial therapeutic strategy for IBD\textsuperscript{33, 34}. Intestinal fungi, although less abundant than bacteria\textsuperscript{35}, also bloom in the inflammatory colonic mucosa and invade the intestinal wall and extra-enteric organs, especially when the host is severely injured or immunocompromised\textsuperscript{20, 36}. However, whether anti-fungal treatment is useful in treating IBD has not been investigated.

In this study, we found that A-DSS colitis in mice can be remarkably promoted by high dose of fluconazole, these results are in line with a recent research showing similar effects of fluconazole treatment on the severity of A-DSS colitis\textsuperscript{37}. Then, we treated A-DSS colitis mice with another broad spectrum anti-fungal drug (AmpB) and interestingly got a similar observation. Amphotericin-B and fluconazole target fungi through quite different mechanisms\textsuperscript{37}, so we put forward the hypothesis that the aggravation of acute DSS-colitis in DSS+fluconazole mice could not be an off-target effect of fluconazole but was like a specific effect of disrupting the intestinal micro-ecological equilibrium.

Gastrointestinal microbiota was considered to be divided into two distinct ecosystems: the mucosal microbiota (adhered to the intestinal epithelium) and the fecal microbiota (mostly present in the stools)\textsuperscript{12}. Compared with fecal microbiota, the mucosal microbiota is believed to be more closely associated with the epithelial innate immune response\textsuperscript{38, 39}; therefore, we focused on the change of mucosal
microbial composition in the gut of normal and anti-fungal drug-treated mice in the current study. Interestingly, both drugs dramatically decreased the mucosal fungal 18S rDNA level in the murine colon. Notably, the mucosal 16S rDNA (bacterial) level in the colon did not change after exposure to anti-fungal drugs. We further detected the prevalence of various intestinal bacteria in the colonic mucosa of normal and fluconazole-treated mice by high-throughput sequencing. As evidenced by the results of PLS-DA (Figure 4B), significant changes occurred in the bacterial composition of fluconazole-treated mice compared with the normal control. We detected eight major genera (*Helicobacter*, *Clostridium* cluster XIVa, *Anaerostipes*, *Lachnospiracea incertae sedis*, *Lactobacillus*, *Mucispirillum*, *Marvinbryantia*, and *Alistipes*) in the colonic mucosa of normal mice (Figure 4D), while thirteen major genera (*Helicobacter*, *Lactobacillus*, *Clostridium* cluster XIVa, *Lachnospiracea incertae sedis*, *Alistipes*, *Marvinbryantia*, *Bacteroides*, *Hallella*, *Xylanibacter*, *Barnesiella*, *Anaerostipes*, *Flavonifractor*, and *Prevotella*) were detected in the fluconazole-treated mice (Figure 4E). Among the major bacterial genera, *Helicobacter*, *Bacteroides*, and *Alistipes* are positively associated with intestinal inflammation and/or tumour burden, while *Clostridium* cluster XIVa, *Lachnospiracea incertae sedis* (both of which are butyrate-producing bacteria), and *Lactobacillus* were demonstrated to be beneficial for IBD patients. Therefore, we quantified these bacteria in the colonic mucosa by real-time PCR. The prevalence of two pathogenic bacterial genera (*Bacteroides* and *Alistipes*) was found to be significant higher, while the levels of probiotic *Clostridium* cluster XIVa and *butyryl-CoA/acetate-CoA transferase* were significantly lower, in fluconazole-treated mice than in the normal control mice, indicating that fluconazole aggravated intestinal inflammation by promoting the proliferation of pathogenic *Bacteroides* and *Alistipes* while reducing the proportion of beneficial butyrate-producing bacteria (e.g., *Clostridium* cluster XIVa, etc.). However, the level of *Helicobacter*, the most abundant genus in the mouse colon, did not significantly differ between the two groups. Interestingly, the level of *Lactobacillus*, a probiotic bacterium, was increased in the colon after fluconazole treatment. Gut inflammation is affected by the total intestinal microbial community, and the strength of the anti-inflammatory effect of *Lactobacillus* in the gut remains to be investigated.

We also compared the levels of the six bacterial genera and *butyryl-CoA/acetate-CoA*
transferase in the colonic mucosa of AmpB-treated mice and normal controls, and observed significantly higher levels of Alistipes and significantly lower levels of Clostridium cluster XIVa and butyryl-CoA/acetate-CoA transferase in the colonic mucosa of AmpB mice than in the normal control (Figure S6), indicating that Alistipes and several butyrate-producing bacteria (e.g., Clostridium cluster XIVa, etc.) might play a role in the progression of intestinal inflammation. However, Helicobacter, Lactobacillus, and Bacteroides did not show obvious differences between the two groups. The pharmacological effect of AmpB is considerably different from that of fluconazole, and whether other bacterial genera affect gut inflammation in AmpB-treated mice remains to be studied.

IBD patients usually exhibit decreased abundance of occludin and ZO-1, two key tight junction proteins that constituting intestinal mucosal barrier and regulate epithelial survival and pathogenesis of gut inflammation and carcinoma, in their intestinal mucosa. In this study, we found that a high dose of anti-fungal drugs (fungi-depleted therapy) significantly downregulated colonic occludin and ZO-1 expression. Previous studies have reported a positive association between butyrate-producing bacteria and tight-junction proteins. Therefore, we believe that the decreased levels of Clostridium cluster XIVa and other butyryl-CoA/acetate-CoA transferase-expressing butyrate producers could play an important role in reducing mucosal tight-junction protein expression, thereby exacerbating acute DSS-colitis in mice.

IBD is a chronic relapsing condition with life-long effects on patients. Fungi are more abundant in the colons of patients with a long history of UC than in those of patients with a short history and intestinal fungi could be broken down by some pathogenic gram-negative bacteria and provide energy for the latter in the gut. Moreover, fungi can translocate into colonic mucosa and extra-enteric organs in C-DSS (but not in normal and A-DSS) murine IBD models and could aggravate disease severity, indicating anti-fungal therapy could be a promising approach to improve the chronic IBD condition. However, only limited clinical studies have reported the anti-fungal treatment in chronic IBD or IBD patients with fungal infections.

Intestinal fungi could play a more important role in affecting chronic intestinal inflammation than
was believed earlier. Properly use of anti-fungal agents in treating IBD could be of great importance, while the appropriate dosage of these agents are need to be verified. In this study, we treated chronic DSS-colitis mice with three different doses of fluconazole, and found that only a moderate dose of fluconazole (0.125 mg/mL) could significantly accelerate chronic DSS colitis, while a lower dosage (0.0625 mg/mL) and higher dosage (0.5 mg/mL) led to no obvious improvements. However, whether this dosage of fluconazole in animal experiment could be extrapolated to clinical applications remains further investigation.

In conclusion, we demonstrated that fungi are important co-factors in maintaining gut microbiological homeostasis. Additionally, we provided some preliminary data for the usage of anti-fungal drugs in treating IBD, and verified the existence of fungal-bacterial interactions in gut inflammation. Further, we demonstrated that too high a dose of anti-fungal agents could agitate the intestinal micro-ecological balance and dampen the intestinal mucosal barrier, only appropriate dose of anti-fungal drugs could be used to relieve DSS-colitis in mice. However, we only observed the effects of different doses of fluconazole on C-DSS (but not A-DSS) murine colitis. Besides, anti-fungal agents used in treating IBD are still lack of quality clinical trial, further studies are also required to clarify the mechanism underlying the regulation of intestinal health by enteric microbiota and investigate whether a specific dose of fluconazole (or other anti-fungal drugs) needs to be used in IBD and whether the anti-fungal drugs should be applied, alone or in combination with other anti-inflammatory drugs (or antibiotics) in the clinical IBD treatment.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

X.Q. designed the research; X.Q., X.L., Z.W., F.Z. and N.W. performed the research; X.Q., X.Y., analysed the data; X.Q. wrote the paper. Y.L. helped edit this manuscript.

References:


14 A. Y. Koh, Current Fungal Infection Reports, 2013, 7, 144-151.


Gastroenterol., 2010, 105, 1820-1829.


Figure 1. Effect of fluconazole in mice with acute-DSS (A-DSS) colitis. Four groups of mice (Normal, A-DSS, Fluconazole, and A-DSS+Fluconazole group) were treated as shown in Table 1. (n = 8–10/group). Weights (A) were measured on days 14–23, and the percent weight change was calculated. Colon lengths (B) and Histological scores (C) were measured on day 23 after sacrifice. The values are expressed as mean ± SEM. Histological sections of colonic tissue stained with hematoxylin and eosin showing the effects of fluconazole in mice. (D) (HE, ×100). *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 2 Inflammatory cytokine (IL-6, IL-17A, and IFN-γ) levels in the colonic mucosa and serum of normal, Fluconazole, A-DSS and A-DSS+Fluconazole mice. (A–C) mRNA expression levels of IL-6, IL-17A, and IFN-γ in the colon were measured by Realtime PCR and normalized to Rpl32 mRNA. (n = 8–10 /group). (D–F) IL-6, IL-17A, and IFN-γ cytokine serum concentrations were measured by MILLIPLEX™ Immunoassays. The values are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 3 Effect of amphotericin B (AmpB) in mice with acute-DSS (A-DSS) colitis. Four groups of mice (Normal, AmpB group, A-DSS group, and A-DSS+AmpB group) were treated as shown in Table 1. (n = 8–10/group). Weights (A) were measured on days 14–23, and the percent weight change was calculated. The A-DSS+AmpB mice exhibited higher weight loss on days 19 and 22 than the DSS mice. Colon lengths (B) and Histology scores (C) were measured on day 23 after sacrifice. The values are expressed as mean ± SEM. Histological sections of colonic tissue stained with hematoxylin and eosin showing the effects of AmpB in mice. (D) (HE, ×100). *P < 0.05, **P < 0.01, ***P < 0.001.  # P < 0.05.
Figure 4  Comparison of intestinal bacterial compositions between Normal and Fluconazole treated mice. (A) The bacterial OTU attribution in colonic mucosa of normal and fluconazole mice. The green circle indicates the OTUs present in normal samples, and the red circle indicates the OTUs present in the fluconazole samples; overlap indicates the OTU shared by both samples. (B) Partial least-squares discriminant analysis (PLS-DA) scores plot based on the relative abundance of bacterial OTUs (97% similarity level) in the colonic mucosa of normal (green triangle) and fluconazole (red triangle) mice. (C) Specific bacterial phylum abundance in the colonic mucosa of normal and fluconazole mice. Major bacterial genera (relative abundance ≥ 0.01 on average) in the colonic mucosa of normal (D) and fluconazole (E) treated mice.
Figure 5  Six different bacterial genera (Bacteroides, Alistipes, Lactobacillus, Clostridium cluster XIVa, Lachnospiracea incertae sedis, and Helicobacter) and butyryl-CoA/acetate-CoA transferase level in the colonic mucosa of normal and fluconazole mice were determined by Realtime PCR, β-actin was used as the internal control. The values are expressed as mean ± SEM. *P < 0.05.
Figure 6. Tight junction proteins (Occludin and ZO-1) expression in colonic mucosa from normal, Fluconazole, and AmpB treated mice. Proteins expression was first analyzed by Realtime PCR (A and B), with Rpl32 mRNA to be the internal control; and then, was confirmed by Western blot analysis, with β-actin protein to be the internal control.
Figure 7. Effect of different concentrations of fluconazole in mice with chronic-DSS (C-DSS) colitis. Mice were randomly divided into C-DSS, C-DSS+Flu1, C-DSS+Flu2, and C-DSS+Flu3 groups. (n = 6–7 / group). All of the mice were treated as was illustrated in Figure S1. Weights (A) were measured on days 43–57, and the percent weight change was calculated. Colon lengths (B) and Histology scores (C) were measured on day 57 after sacrifice. The values are expressed as mean ± SEM. Histological sections of colonic tissue stained with hematoxylin and eosin showing the effects of different dose of fluconazole in mice. (D) (HE, ×100). *P < 0.05.
Figure 8. Inflammatory cytokine levels in the colonic mucosa and serum of C-DSS, C-DSS+Flu1, C-DSS+Flu2, and C-DSS+Flu3 mice. (A–C) IL-6, IL-17A, and IFN-γ mRNA expression levels in the colon were measured by Realtime PCR and normalized to Rpl32 mRNA. (n = 6–7/group). (D–F) IL-6, IL-17A, and IFN-γ cytokine concentrations in serum were measured by MILLIPLEX™ Immunoassays. The values are expressed as mean ± SEM. *P < 0.05.
Mice were treated with different drinking water regimens ad libitum (see Materials and Methods). All mice were sacrificed on day 23. Colonic mucosal samples were collected and detected. N water: normal water. Flu water: N water containing 0.5 mg/mL fluconazole. ING-B: intragastric administration of amphotericin B (AmpB) (1 mg/kg bodyweight).

Table 2 Primers used for the pro-inflammatory cytokines (IL-6, IL-17A, and IFN-γ) detecting. Rpl32 was used as the housekeeping gene.

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<th>Amplicon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>TGATGCACCTTGCAGAAAACA</td>
<td>ACCAGAGGAAATTTCATAGGC</td>
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<tr>
<td>IL-17A</td>
<td>CAGGAGCAGCAAAACATGA</td>
<td>GCAACAGCATCAGAGACACAGAT</td>
</tr>
<tr>
<td>IFN-γ</td>
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<td>GCTTCTGAGGCTGAGATTC</td>
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<tr>
<td>Occludin</td>
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</tr>
<tr>
<td>ZO-1</td>
<td>AGGACACCAAAACATGTGAG</td>
<td>GSCATTCTGCCTGCTGACATA</td>
</tr>
<tr>
<td>Rpl32</td>
<td>AAGCGAAACTGGCGGAAAC</td>
<td>TAACCGATGTTGGCATCAG</td>
</tr>
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</table>

Table 3 Primers for detection of intestinal total fungi (18S rDNA), bacteria (16S rDNA) and several specific bacteria related to IBD at the genus level. β-actin was used as an internal standard.

<table>
<thead>
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<th>Amplicon</th>
<th>Forward primer (5´–3´)</th>
<th>Reverse primer (5´–3´)</th>
</tr>
</thead>
<tbody>
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<td>16S (341F &amp; 534R)</td>
<td>ATTACCCGGGCTGCTGGG</td>
<td>ATTACCCGGGCTGCTGGG</td>
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<tr>
<td>18S</td>
<td>ATTGAGGGGCAAGTCTGTTG</td>
<td>CGGATCCTCAGTCGCGGATAG</td>
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<tr>
<td>β-actin</td>
<td>ATGACCCAGATCATGTGTGA</td>
<td>TACGACCAGAGGACTACAG</td>
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<tr>
<td>Bacteroides</td>
<td>CTGAAACCAGGCAAAGTACGG</td>
<td>CGGAAAATTTCACAACGACTTA</td>
</tr>
<tr>
<td>Alistepes</td>
<td>TTAGAGATGGGCATGCATTGT</td>
<td>TGAATCCCTCGGTATT</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>GCAGCACTGGGAAATCTCCACAA</td>
<td>GCTCGTTTACGCCCAAT</td>
</tr>
<tr>
<td>Organism</td>
<td>Forward Sequence</td>
<td>Reverse Sequence</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Clostridium cluster XIVa</td>
<td>AAATGACGGTACCTGACTAA</td>
<td>CTTTGAGTTTCATTCTTGCGAA</td>
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<tr>
<td>Lachnospiracea incertae sedis</td>
<td>GYGAAGAAGTATTTCGGTAT</td>
<td>CCAACACCTAGTATTTCATC</td>
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<tr>
<td>Butyryl-CoA/acetate-CoA transferase</td>
<td>GCIGAICATTTCCITGGAAYWSITGG</td>
<td>CCTGCCTTTGCAATRTCICRAANG</td>
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<tr>
<td>Helicobacter</td>
<td>ACCAAGGC(A/T)ATGACGGGTATC</td>
<td>CGGAGTCTAGCCGGTGCTTATT</td>
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