Gut microbiota-derived 5-hydroxyindole-3-acetic acid mediates the anti-colitis effect of Phellinus gilvus in mice

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Research

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Abstract

**Background:** The gut microbiota plays crucial roles in various diseases and mediates the therapeutic effect of many drugs.

**Results:** In this study, we found that ingestion of *Phellinus gilvus* extract (SH), a traditional Chinese medical mushroom, effectively ameliorated dextran sodium sulfate (DSS)-induced colits in mice, and the gut microbes were essential for its therapeutic effect. The enrichment of *Alistipes* spp. especially *A. onderdonkii* was highly associated with the amelioration of colitis by SH, and oral gavage of live *A. onderdonkii* potently and strain-specifically mitigated DSS-induced colitis in mice. 5-Hydroxyindole-3-acetic acid (5HIAA), a structural analogue of a known aromatic hydrocarbon receptor (AhR) activator indole-3-acetic acid (IAA), was significantly enriched after SH treatment and exhibited more potent anti-colitis efficiency than IAA in mice.

**Conclusion:** Our results demonstrated the gut microbiota-derived 5HIAA could be an effective candidate in treatment of colitis, and modulation of the gut microbiota-indole derives-AhR pathway may be a novel strategy in development of next-generational anti-colitis drugs.

**Introduction**

Inflammatory bowel disease (IBD) with the two main forms of ulcerative colitis (UC) and Crohn's disease (CD) affects approximately 0.5% population in the developed countries and has increased the incidence in developing countries [1]. The typical symptoms of IBD include a urgent diarrhea, intermittent abdominal pain, rectal bleeding, and weight loss, all of which significantly depreciate life quality of affected individuals and increase a risk in developing colon cancer [2]. To date, IBD is not medically curable [3] and treatment options currently available for UC include surgical resection of the affected colon, while treatment of all IBDs includes drug, nutritional, and dietetic therapies [3, 4]. A number of studies have shown that altered and imbalanced composition of the gut microbiota or microbial dysbiosis contributes to IBD progression; for example, microbial metabolites, like short-chain fatty acids (SCFAs) and indole derivatives, are the key factors in pathogenesis of colitis [5, 6]. Butyrate-producing bacterial species, like *Faecalibacterium prausnitzii* that belongs to the *Clostridium* IV class, have been previously reported to associate with IBD development [7], while administration of butyrate was able to effectively alleviate symptoms of UC patients [8]. Furthermore, both animal and patient studies showed that downregulation of the aryl hydrocarbon receptor (AhR) promoted colitis development [9], while AhR agonists possessed a potent effect against colitis through increase in Interleukin (IL)-22 [10]. The gut microbiota-derived indole derivatives were the major ligands to activate AhR, which may indicate microbial metabolites as a potential reservoir to develop critical preventive and therapeutic agents against IBD [4]. Since IBD is a group of inflammatory conditions of the colon and small intestine and the cause are also multifactorial [11], further study of IBD novel molecular mechanisms could lead to the effective approaches in IBC treatment.
Traditional Chinese Medicine (TCM), successfully used in treatment of various human diseases for thousands of years, can modulate composition of the gut microbiota by promoting probiotics and decreasing pathogens to ultimately prevent development and progression of colitis and other diseases [12]. Increasing evidence has recently acknowledged the effects of *Phellinus gilvus* (also named as mulberry Sanghuang, SH) with anti-inflammation [13, 14], anti-tumor [15–17], and anti-oxidation [14] properties. SH was also reported to modulate growth, immunity, and fecal microbiota level in pigs [18], while other two species, *P. linteus* and *P. igniarius*, have been also reported to effectively ameliorate colitis [19, 20]. Nevertheless, the effect of *P. gilvus* on colitis and the behind mechanisms governing the therapeutic effects of Sanghuang is yet unclear.

In this study, we investigated the anti-colitis effects of SH using a mouse model of colitis *in vivo* and then explored the underlying molecular events. We assessed the anti-colitis activity of SH and confirmed the necessity of gut microbes in SH’s therapeutic effect, then identified key species (*Alistipes onderdonkii*) and its active metabolite (5-hydroxyindoleacetic acid (5HIAA)) that may mediate the colitis-ameliorating action of SH, and speculated the potential molecular mechanism (AhR activation) of the active gut bacterial metabolite. Upon completion of our experiments, we expected to provide insightful information regarding gut microbe-derived 5HIAA in control of colitis.

**Results**

**SH ameliorates DSS-induced colitis**

In this study, we first established a mouse model of colitis (Fig. 1) and orally administrated a low or high dose of SH to the mice. We found that SH significantly ameliorated DSS-induced colon colitis in terms of body weight loss, disease activity index (DAI), colon length and colonic histology (Fig. 1a, c-g). Furthermore, this colitis mouse model also showed that DSS in the drinking water significantly downregulated expression of various tight junction genes (*Occludin, Claudins*-2, -3, -4, and *ZO-1*) and proteins (*Occludin, Claudins*-3, and -4; Fig. 2a-c), and substantially diminished mucin-producing goblet cells (Fig. 2d) in the mouse colons. However, SH treatment evidently restored expression of occludin and claudin-2, -3 and -4 mRNA (Fig. 2a) and proteins (Fig. 2b, c). SH treatment also dramatically induced the number of goblet cells (Fig. 2d).

After that, we assessed the role of the gut microbiota in colitis development by established pseudo-germ-free mice with daily oral administration of a large dose of antibiotics (ampicillin + norfloxacin, 300 mg/kg) to compare the colitis-mitigating efficacy of SH. We found that administration of antibiotics exhibited a comparable effect on the symptoms of colitis, including improvement of the mouse weight loss, shortened colon length, and colonic histology in DSS + antibiotics vs. DSS-only groups of mice (Fig. 1b-g). The antibiotics treatment showed a weak effect on improvement of the DAI score (Fig. 1c), expression of the tight junction genes (except *Claudin*-4 mRNA level) (Fig. 2a, b, c), and goblet cells number (Fig. 2d). However, combination of SH with antibiotics treatment had no any synergic effects on body weight, colon length, colonic histology and tight junction gene transcription in DSS + antibiotics +
SH vs. DSS + SH groups of mice (Fig. 1b-g and Fig. 2a), but had a deteriorated effect on the DAI score, expression of tight junction proteins, and goblet cells number in DSS + antibiotics + SH vs. DSS + SH groups of mice (Fig. 1c and Fig. 2b, c, d).

**Alleviation Of Colon Inflammations After Sh Treatment**

A previous study reported SH effect on inhibition of inflammation [13]. In this study, we quantified level of different cytokines in the colon tissues and sera and found that SH treatment significantly reduced level of pro-inflammatory factor mRNAs, including $\text{TNF}_\alpha$, $\text{IL}-1\beta$, $\text{IL}-6$, and $\text{IL}-17A$, and chemokine mRNAs ($\text{CXCL}-1$, $\text{CCL}2$, and $\text{MCP}-1$) but enhanced level of the anti-inflammatory factor mRNAs, such as $\text{IL}-4$, $\text{IL}-10$, and $\text{IL}-22$ in the colon tissues (Fig. 3a, b, c) or in the sera (Fig. 3d, e). Furthermore, administration of antibiotics also significantly decreased level of $\text{TNF}_\alpha$ mRNA in the colon and improved serum mRNA level of these pro-inflammatory and anti-inflammatory factors except $\text{IL}-1\beta$. As expected, the combined SH and antibiotics had no additional beneficial effects on regulation of these cytokine expressions (Fig. 3).

**Alistipes mimic of SH anti-colitis effect**

We next explored the SH effect on the gut microbiota in both conventional and antibiotics-induced pseudo-germ-free mice. Specifically, we extracted DNA and mRNA samples from the cecal contents and performed both 16S rRNA gene amplicon and 16S rRNA mRNA pyrosequencing to reveal the compositional alterations in total (reflected by gene amplicon sequencing) and alive (reflected by mRNA sequencing) gut bacteria. Our mRNA-based metatranscriptomic analysis showed that SH treatment of mice had a shifted alive gut microbial community vs. the DSS only group, while this shift was disappeared after addition of antibiotics (Fig. 4a, b). The cluster analysis of the main genera revealed that mice treated with SH were clustered with normal animals and departed from DSS only treatment without addition of antibiotics, while the SH + DSS group was clustered with DSS only group after their gut microbes were inhibited by antibiotics (Fig. 4c).

Furthermore, according to the taxon-based analysis, nine genera, including *Alistipes*, *Clostridium* IV, and *Butyricicoccus*, were enriched more than 1.5 folds after SH treatment, whereas co-treatment of mice with antibiotics dramatically decreased or even diminished these bacteria populations (Fig. 4d, e). Our correlation analysis of individual genus and colitis indicators showed that *Alistipes* and *Clostridium* IV were significantly associated with colon length and the DAI at the top 2 rankings (Fig. 4f). These findings suggest that SH treatments altered the composition of the intestinal bacterial populations, mainly by elevating the abundance of *Alistipes* and *Clostridium* IV, which may be beneficial to inhibiting DSS-induced colitis. These data were reproduced by DNA pyrosequencing (Supplementary Fig. 1), revealing a similar modulation by SH treatment in both total and alive gut microbes.

**Alistipes onderdonkii alleviation of DSS-induced colitis**

A recent study showed that *Alistipes* from the gut contains many anti-inflammatory bacteria and its is beneficial in prevention and amelioration of colitis [21, 22]. In this study, we found that *A. onderdonkii* was considerably enriched after SH treatment at the genus and species levels (Fig. 5a, b). Thus, *A.
onderdonkii may be a key mediator for SH anti-colitis action. To confirm it, we selected three different A. onderdonkii strains to assess their anti-colitis activity in the mouse model of colitis and found that two of these three strains significantly improved mouse body weight loss and shortening colon length, DAI index, and colonic histology (Fig. 5c-g). The adverse effect of DSS on regulation of the serum cytokines was also clearly restored by A. onderdonkii (Fig. 5h), indicating that A. onderdonkii is an effective gut bacterium to possess the anti-colitis effect or mediate the effect of SH. However, the colitis-alleviating effect of A. onderdonkii exhibited obvious species specificity, as oral administration of one A. onderdonkii strain led to more severe colitis (Supplementary Fig. 2).

5-hydroxyindole-3-acetic Acid (5hiaa) As A Sh-enriched Gut Microbial Metabolite

To further elucidate the mechanisms of SH action to prevent colitis, we carried out a metabolomics analysis of mouse cecal materials to identify the key metabolites of the gut microbiota that associated SH anti-colitis effect. We found that SH treatment significantly changed the metabolomics of mouse cecal materials but such a change was substantially deteriorated by antibiotics treatment (Fig. 6a, b). Specifically, DSS treatment significantly reduced level of six metabolites, i.e., adenine, 2-isopropylmalic acid, 2(1H)-quinolinone, luminchrome, 5-hydroxyindole-3-acetic acid, and equol, whereas SH treatment markedly restored them (Fig. 6c). Our correlation analysis of individual metabolite and pathological indexes of colitis, including the DAI and colon length, revealed that 18 metabolites were associated with colitis remission, among which quinolinone and 5HIAA were the top 2 compounds (Fig. 6d). 5HIAA is a known gut microbial metabolite and a previous study demonstrated that the gut microbes-derived indole-3-acetic acid (IAA), a closely related compound to 5HIAA, possessed an anti-colitis activity [23]. In this study, we found that the IAA level was rather low, but 5HIAA was high; thus, we speculated that 5HIAA may be the effective anti-colitis metabolite. Our correlation analysis of 5HIAA and the gut bacteria showed that 5HIAA level was associated with the abundance of Alistipes and Clostridium IV, suggesting that they could be potential 5HIAA producers (Fig. 6e). In addition, we further quantified the level of 5HIAA in the spent culture supernatants of A. onderdonkii using the high-performance liquid chromatography (HPLC), and found that the 5HIAA concentration in A. onderdonkii spent culture medium was 33.5 µg/mL high, indicating that A. onderdonkii is a key producer of 5HIAA (Supplementary Fig. 3).

5hiaa Suppresses Colitis

To further confirm the effect of 5HIAA on suppression of colitis, we treated the mouse model of colitis with an equal dose of 5HIAA or IAA. Our data showed that oral administration of 5HIAA significantly improved mouse body weight loss and shortening colon length (Fig. 7a, b, c), while the DAI index and colonic pathological damage were also remarkably improved after 5HIAA treatment (Fig. 7d, e). Compared with IAA, 5HIAA was more potent (Fig. 7a-e). 5HIAA also efficiently increased expression of colonic tight junction genes, including occludin, claudin-2, -3, and − 4 mRNAs (Fig. 7f-h).

We also found the similar anti-inflammatory effect of 5HIAA on colitis as aryl hydrocarbon receptor (AhR) activator indole-3-acetic acid (IAA). A previous study has reported that the indole derivatives ameliorated colitis through activation of the aryl hydrocarbon receptor (AhR) and suppression of pro-inflammatory
cytokines/chemokines such as TNF-α, IFN-γ, MCP-1, and IL-17 but promotion of anti-inflammatory factors such as IL-10 and IL-22\textsuperscript{5,23}. In this study, 5HIAA had a similar anti-inflammatory effect with IAA on colitis. We found that both 5HIAA and IAA were able to remarkably reduce expression of TNF-α, IL-1β, IL-6, IL-17A, CXCL-1, CCL2, and MCP-1, but increase transcriptions of IL-4, IL-10 and IL-22 in the colon tissues (Fig. 8a, b, c). Consistently, the dysregulated serum levels of pro-inflammatory and anti-inflammatory factors in DSS-induced colitis mice were significantly reversed by 5HIAA and IAA treatment (Fig. 8d, e).

To assessed the effects of SH and 5HIAA on activation of the AhR pathway genes, we found that SH or 5HIAA treatment did not significantly induce level of AhR mRNA, but activated the transcription of its downstream genes, including CYP1A1, CYP1A2 and COX2 mRNAs in the mouse abdominal macrophages (Supplementary Fig. 4), suggesting an activating response of the AhR pathway after administration of SH and 5HIAA to the macrophages. Similarly, SH and 5HIAA treatment was able to slightly enhance level of AhR mRNA in the mouse colons (Fig. 9a, b) but significantly induce transcription of AhR downstream genes CYP1A2 and CYP1B1 mRNAs (Fig. 9c-f).

**Discussion**

A great number of previous studies have demonstrated that the gut microbiota or modulation of the gut microbiota associated with development or control of colitis \textsuperscript{[24–28]}, although the precise knowledge regarding the specific bacterial species and the active metabolites that can be used for the clinical management of colitis remains to be defined. In the current study, we not only demonstrated the key role of the gut microbes and metabolites in mediation of SH anti-colitis effect in a mouse model of colitis, but also identified *A. onderdonkii* as a novel beneficial microorganism that possessed an anti-colitis activity \textit{in vivo}. We further found that 5HIAA, an AhR activator and produced by *A. onderdonkii*, is an active metabolite with a more potent anti-colitis effect than indole-3-acetic acid (IAA). Our results revealed that the gut microbiota-indole derivatives-AhR signaling could be used as a novel target in development of next-generational anti-colitis drugs.

Accumulating evidence acknowledged that SH extracts possessed medical usage against inflammation or cancer; for example, the whole-genome sequencing of *Phellinus gilvus* (mulberry Sanghuang) reveals the unique medicinal values \textsuperscript{[15]}, SH impacted growth, immunity, and fecal microbiota in pigs \textsuperscript{[18]}. Moreover, SH possessed antioxidant and anti-inflammatory activities \textit{in vitro} \textsuperscript{[14]} by prevention of intraperitoneal adhesions and abscesses in a rat peritonitis model \textsuperscript{[29]}, while SH extracts suppressed pulmonary inflammation induced by lipopolysaccharide in rats \textsuperscript{[13]}. SH was also able to inhibit melanoma cell growth in mice \textsuperscript{[16]} and induce murine B16-F10 cell arrest at the G0/G1 phase of cell cycle as well as apoptosis \textsuperscript{[17]}. In the current study, we found that SH had an anti-colitis effect in a mouse model of colitis, further confirming the SH anti-inflammation activity. Furthermore, we found that the SH anti-colitis activity was substantially abolished when concomitant with antibiotics. Although antibiotics administration is a common treatment option for colitis and antibiotics alone exhibit potent effect on various colon pathological indexes, such as colon length, histopathology, and cytokines expression, but it showed a quite weak effect on the DAI score, tight junction factors (except *Claudin*-4 mRNA level) and
goblet cell number. Thus, elimination of bacteria alone using antibiotics cannot promote restoration of damaged intestinal epithelial barrier. However, oral SH administration was able to significantly improve the gut barrier by enhancing expression of the tight junction genes and restoring mucin-producing goblet cells. These SH effects were associated with presence of the gut microbiota, whereas these beneficial SH effects were substantially diminished after addition of antibiotics that eliminated the gut microbes. Although germ-free animals are still needed to prove the necessity of gut bacteria in the anti-colitis action of SH, these results suggest that SH may exert its therapeutic action on colitis via, at least partially, modulation of the gut microbiota.

Indeed, the disruption of and imbalanced composition of the gut microbiota, also called gut dysbiosis, has been recently recognized as a key pathogenic factor in colitis development [30]. In IBD, the gut microbiota was associated with expansion of pro-inflammatory bacteria like *Enterobacteriaceae* and *Fusobacteriaceae* and reduction of anti-inflammatory species such as butyrate-producers within the *Lachnospiraceae* family [31, 32]. In the current study, we identified four genera, namely *Clostridium IV, Alistipes, Butyricicoccus*, and *Lachnospiraceae_incertae_sedis*, to be positively associate with colitis alleviation in mice, all of which are known as SCFA-producing bacteria and have been broadly reported as beneficial microbes in colitis management [33, 34]. As a genus in the family of *Rikenellaceae, Alistipes*, a relatively novel genus of bacteria, are highly relevant in dysbiosis and disease because *Alistipes* contain enzymes that can degrade cellulose, while loss of cellulose changed the microbial metabolome, gut immune response, gene expression in intestinal epithelial cells and the mice had an increased sensitivity to colitis induction [22, 35], while the gut dysbiosis plays a role in determination of the compositional *Alistipes* abundance in the feces and associated with correlate with human health [22]. Previous studies also showed that colitis was associated with increase in *Alistipes* abundance [36] and *A. finegoldii* could activate the IL-6/STAT3 signaling to promote colitis-associated cancer development in IL-10−/− mice [37]. However, more recent studies recognized *Alistipes* as SCFA-producing anti-inflammatory bacteria and associated with colitis alleviation [21, 38]. Dziarski et al demonstrated that oral gavage of *A. finegoldii* was able to effectively attenuate mouse colitis [38]. In our current study, we revealed that the abundance of *Alistipes* in both total (16S rDNA-based analysis) and active (16S rRNA-based analysis) gut microbial communities was largely reduced in the mouse model of DSS-induced colitis, whereas SH treatment showed little influence on *Alistipes* level after assessed the composition of total gut bacteria (alive and dead bacteria) using the 16S rDNA-based metagenomics. However, SH treatment significantly increased the abundance of *Alistipes* after assessment of the active gut microbiota (alive bacteria only) using the 16S rRNA-based metatranscriptomics. Thus, such a contradictory effect of *Alistipes* on colitis development should be further evaluated and confirmed. Our current results revealed that oral administration of alive *A. onderdonkii* effectively prevented DSS-induced mouse colitis *in vivo*, suggesting the beneficial effect of *Alistipes* spp. on colitis management. Furthermore, we found that the effect of *A. onderdonkii* on colitis was strain-specific because another *A. onderdonkii* strain was lead to more severe colitis in our mouse model of colitis.
In addition, indole compounds are broadly produced by both gram-positive and -negative bacteria [39]. Recent studies demonstrated that indole derivatives of different origins were potent to inhibit colitis, indicating of a tremendous potential in treatment of colitis [5, 40]. Our current study revealed that SH treatment significantly induced 5HIAA production and 5HIAA treatment was able to significantly suppress DSS-induced colitis in mice. Furthermore, it was widely reported that indole compounds, as known ligands for AhR, could effectively suppress level of serum inflammatory cytokine/chemokines, like TNFa, IFN-γ, MCP-1, IL-17 in colitis [41], enhance expression of IL-10 and IL-22 [5, 42], and strengthen the intestinal epithelial cell barrier [5]. In our current study, treatment with SH, alive A. onderdonkii, and 5HIAA, all significantly suppressed level of pro-inflammatory factors, including IL-1β, IL-6, IL-17, TNFa, and MCP-1, but increased level of anti-inflammatory factors (IL-10, IL-22) and expression of the tight junction genes, e.g., Occludin and Claudins. However, antibiotics treatment only eliminated the serum IL-22 level, which showed a closest correlation with the gut microbes. Although both SCFAs and indoles were reported to be able to enhance IL-10, only indoles can increase IL-22 mRNA [5, 43]. Therefore, the increase in IL-22 expression induced by SH may mainly attribute to 5HIAA induction in the mouse model of colitis. Future investigation will assess the underlying molecular mechanism of SH-induced IL-22 expression; for example, whether this regulation is dependent on AhR activation.

**Conclusion**

Our results demonstrate that modulation of the gut microbiota, especially A. onderdonkii, is a novel mechanism underlying the anti-colitis effect of *P gilvus* extract. The gut microbiota-indole derivatives-AhR axis plays an important role in host immunity, while 5HIAA will be further evaluated as a promising agent in control of colitis.

**Materials And Methods**

**SH preparation**

The fruiting body of cultured *P. gilvus* was obtained from Sericultural Research Institute, Zhejiang Academy of Agricultural Science (Hangzhou, China) and naturally dried in the air and then extracted by boiling in water for 2 h. After that, the aqueous extract was concentrated and mixed with three volumes of ethanol. After centrifugation, the supernatant was collected and concentrated using rotary evaporation and lyophilized in a freeze vacuum and then collected, weighed, and stored at -20°C until use. Our procedure was bale to make one kilogram of *P. gilvus* fruiting body to approximately 125.7 grams of SH, while the polyphenol content could yield 47.77% as determined by a colorimetric method\textsuperscript{15}.

**Animals And Experiments**

Male C57BL/6 mice of 6-weeks of age were purchased from Shanghai Experimental Animal Center (Shanghai, China) and acclimatized under standard animal care conditions for a week under controlled temperature and humidity and alternating 12-hour light and dark cycles. The mice will receive normal mouse chow and be allowed to drink sterile water ad libitum. To assess the anti-colitis effect of SH in the
mouse model of colitis, we randomly divided 80 mice into two main groups, i.e., Group 1 (with no antibiotics supplementation) and Group 2 (with antibiotics supplementation, i.e., ampicillin + norfloxacin, 300 mg/kg/day, + Abs). These two groups of mice were then further divided into four subgroups (n = 10), namely Normal (or Abs), dextran sodium sulfate (DSS), DSS + a low dose of SH (SHL) and DSS + a high dose of SH (SHH). The normal or Abs group was administered an equal volume of the drinking water, the DSS group was established chronic colitis by supplementation of DSS (molecular weight 36–50 kDa) from MP Biologicals (Santa Ana, California, USA) in the drinking water (1.0% w/v) on day 1–5, 8–12, 15–19, 22–26, 29–33, and 36–42. The DSS + SHL and DSS + SHH groups were established colitis and treated with a low or high dose of SH (250 and 400 mg/kg/day, respectively). During the experiments, mouse body weight was measured every 3 days. The disease activity index (DAI) was evaluated on Day 42 (the end of experiments). In the end of the experiment, mice were fasted overnight and blood samples were collected for assessment of different parameters, while the colon tissues were resected and fixed in 4% paraformaldehyde for histological analysis or frozen at -80 °C for biochemical measurements.

Furthermore, we also assessed the anti-colitis effect of 5-hydroxyindoleacetic acid (5HIAA) and indoleacetic acid (IAA) in the mouse model of colitis by divided 40 male C57BL/6 mice of 6-weeks of age randomly into four groups (n = 10), i.e., Negative control (NC), DSS-only, DSS + 5HIAA, and DSS + IAA groups. The NC was given an equal volume of the distilled water, while the other three groups of mice were first established chronic colitis with DSS supplementation in the drinking water (1.0% w/v) (see details in the previous paragraph) and the DSS + 5HIAA and DSS + IAA groups were added treatment with 5HIAA (5 mg/kg) and IAA (5 mg/kg) orally, respectively. During the 42-day experimental period, mouse body weight was measured every 3 days. The disease activity index (DAI) was evaluated on Day 42. In the end of the experiment, mice were fasted overnight and blood samples were collected for analysis and the colon tissues were resected and fixed in 4% paraformaldehyde for histological analysis or frozen at -80 °C for biochemical measurements.

In addition, we assessed the anti-colitis effect of *Alistipes onderdonkii* in mice using three human feces-derived *Alistipes onderdonkii* strains with different genome structures, namely *A. onderdonkii*-1, *A. onderdonkii*-2 and *A. onderdonkii*-3. However, due to the limited amount of alive *A. onderdonkii* microbes we could have collected, we were only able to evaluate their anti-colitis effect in the mouse model of acute colitis. In particular, we divided 40 male C57BL/6 mice of 6-weeks of age randomly into four groups (n = 10), i.e., NC, *A. onderdonkii*-1, *A. onderdonkii*-2, and *A. onderdonkii*-3. The NC was given an equal volume of the YCFA bacterial culture medium, while the other three groups were orally gavaged with respective *A. onderdonkii* strain (10⁹ CFU/animal per day). From the fourth day after treatment, all animals were simultaneously given DSS in the drinking water (3.0% w/v) for additional seven days. Mouse body weight and the disease activity index (DAI) were assessed every day and in the end of the experiment, mice were fasted overnight and blood samples were collected for analysis and the colon tissues were resected and frozen at -80 °C for biochemical measurements.

**Quantitative Reverse Transcriptase-polymerase Chain Reaction (qrt-pcr)**
Total cellular RNA was isolated from mouse colonic tissues using the Trizol reagent (Thermo-Fisher, Waltham, MA, USA) and reversely transcribed into cDNA using the Superscript Preamplification System (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturers’ instructions. qPCR was then amplified using the SYBR Green PCR mix (Thermo Fisher) according to the manufacturer’s protocol in a StepOnePlus real-time PCR system (Applied Biosystems, Foster city, CA, USA). Level of β-actin mRNA was used as the internal control and primer sequences used to amplify each gene are listed in Table S1.

**Histology And Immunofluorescence**

Length of the entire colons of each mouse was measured and recorded after tissue resections and a segment of the colon tissues from each mouse was taken and fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 5 µm-thick sections. Multiple sections from consecutive sections were stained with hematoxylin and eosin (H&E) or Alcian blue for histology assessment under a light microscopy (Nikon Co., Japan).

For immunofluorescence, the rehydrated tissue sections were blocked in 5% bovine serum albumin (BSA) for 1 h and incubated overnight at 4°C with different primary antibodies, i.e., a mouse anti-Occludin antibody (Cat. #66378-1-Ig; 1:100; Proteintech, Rocky Hill, NJ, USA) or a rabbit anti-Claudin-3 antibody (Cat. #16456-1-A; 1:100; Proteintech). The sections were then washed with phosphate buffered saline (PBS) and incubated for 1 h at room temperature with a secondary antibody [i.e., The Alexa Fluor 647-conjugated donkey anti-mouse IgG (Cat. #ab150107 and the lot #GR292574-3; Abcam, Cambridge, MA, USA) or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Cat. #ab150073 and the lot #GR3191541; Abcam), both 1:250]. The DAPI-containing medium (Cat. #C1002, Beyotime, Shanghai, China) was used to counterstain the cell nuclei. The immunostained sections were revised and scored under a fluorescent microscope (Nikon Eclipse 80i).

**Western Blot**

Homogenized tissues were lysed in a radioimmunoprecipitation assay buffer (RIPA; Cat. #R0020, Solarbio, Beijing, China) containing protease inhibitor cocktails (Cat. #B14001; Bimake, Houston, Texas, USA). The each amount of these protein samples was resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). For Western blotting, the membranes were blocked in 5% non-fat milk for 1 h at the room temperature and then incubated with a primary antibody at 4°C overnight. The primary antibodies were antibodies against Occludin (Cat. #13409-1-AP; Proteintech), Claudin 3 (Cat. #16456-1-AP; Proteintech), Claudin 4 (Cat. #16195-1-AP; Proteintech), E-cadherin (Cat. #20874-1-AP; Proteintech), and β-actin (Cat. #K200058M, Solarbio). After that, the membranes were washed with PBS-Tween 20 (PBS-T) and then incubated with a secondary antibody [Alexa Fluor 790-conjugated goat anti-rabbit IgG (Cat. #A11369 and the Lot #1152220; Invitrogen, Carlsbad, California, USA), 1:10000]. The positive protein signals were visualized by the chemiluminescence imaging system (Amersham Imager 600, GE
Healthcare Life Sciences) and the relative intensity of protein bands was quantified using Image J software (National Institute of Heath, Bethesda, MD, USA).

**Metabolomic Analysis**

The fecal samples from each mouse were collected, snap-frozen in liquid nitrogen, and stored at -80 °C. Fecal DNA/mRNA extraction, PCR amplification and rDNA/rRNA pyrosequencing at the V4 - V5 regions were performed by Majorbio BioTech Co., Ltd. (Shanghai, China) as previously described [44].

Metabolomics analysis of fecal samples was performed according to a previous study [45]. Briefly, the stool samples were collected and underwent the untargeted metabolomics profiling using the UHPLC system (Genedenovo, Guangzhou, China).

**Statistical analysis**

The data were expressed as the mean ± SEM and analyzed using the one-way analysis of variance (ANOVA) test and Dunnett's test (as a post hoc test) to compare differences among groups for pharmacological parameters. For metagenomic and metatranscriptomic analysis, the alpha diversity of the microbiome was calculated based on the operational taxonomic units (OTU) level using the Mothur software (version 1.30.1) [46]. The principal component analysis (PCA) and principal coordinate analysis (PCoA) were performed using the R and visualized by the R package (R Core Team) [47], whose significant differences were evaluated by the Adonis analysis in R. The P-values were adjusted for multiple comparisons using the Benjamini and Hochberg. False discovery rate and significance was set at $q < 0.05$. A $p$-value $< 0.05$ was considered to be statistically significant.

**Abbreviations**

AhR: Aromatic hydrocarbon receptor; CD: Crohn's disease; DAI: Disease activity index; DAPI: 4’, 6-Diamidino-2-phenylindole; DSS: Dextran sodium sulfate; 5HIAA: 5-Hydroxyindole-3-acetic acid; IAA: Indole-3-acetic acid; IBD: Inflammatory bowel disease; IL: Interleukin; NC: Negative control; OUT: operational taxonomic units; PBS: Phosphate buffered saline; PCA: Principal component analysis; PCoA: principal coordinate analysis; PVDF: Polyvinylidene difluoride; SCFAs: Short-chain fatty acids; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SH: Mullberry Sanghuang; TCM: Traditional Chinese Medicine; UC: Ulcerative colitis.

**Declarations**

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**Authors’ contributions**
Yougui Li and Chongming Wu contributed to the conception of the study. Yuqing Sun, Shi Zhong, Jinxing Huo, Le Sun, Fang Zhang and Jianxun Zhu performed the experiments. Yougui Li and Chongming Wu analyzed the data and prepared the manuscript.

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**Availability of data and materials**

Up on request.

**Ethics approval and consent to participate**

Our animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Academy of Agricultural Sciences (#ZAAS-2017051, #ZAAS-2018046, and #ZAAS-2019044) and followed the Guidelines of the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declared that there is no conflict of interest in this work.

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Figures

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Phellinus giglus extract (SH) amelioration of DSS-induced mouse colitis. a and b, Mouse body weight curves in conventional (a) and antibiotics-induced pseudo-germ-free (b) mice. c, The DAI score. d, Macroimagines of the colon. e, Length of the colons. f, Histology of the colons. g, The histology score of the colons. Antibiotics used were a combination of ampicillin and norfloxacinc (300 mg/kg/day). The data are the mean ± sem (n=10). N.S., no significant. *P<0.05, **P<0.01, and ***P<0.001 using one-way ANOVA with Dunnett’s test as a post hoc test.
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Phellinus gilvus extract (SH) improvement the intestinal epithelial barrier. a and b, levels of colon tight junction factor mRNA (a) and protein (b). c, Immunofluorescence of claudin 3 expression in mouse colon tissues. d, Alcian blue staining of mouse colon tissues. The antibiotics used were a combination of ampicillin and noroxacin (300 mg/kg/day). The data are the mean ± sem (n=10). N.S., no significant. *P<0.05, **P<0.01, and ***P<0.001 using one-way ANOVA with Dunnett’s test as a post hoc test.
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Phellinus gilvus extract (SH) modulation of alive gut microbiota. a and b, The principal component analysis (PCA) (a) and weighted unifrac principal coordinate analysis (PCoA) (b). c, Hierarchical cluster based on the Manhattan distance. d and e, The main genera increased after SH treatment in conventional (d) and pseudo-germ-free mice (e). f, The correlation between genus and colitis indicators. The data are the mean ± sem (n=10). *P<0.05, **P<0.01, and ***P<0.001 using one-way ANOVA with Dunnett’s test as a post hoc test.
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Alistipes onderdonkii prevention of DSS-induced mouse colitis. a and b, The relative abundance of Alitipes (a) and A. onderdonkii (b) in mouse feces. c and d, Mouse body weight curve (c) and body weight change (d) during the experiment. e and f, The DAI score curve (e) and the area under the curve (AUC; f). g, The colon histology. h, Serum levels of inflammatory cytokines. The data are the mean ± sem (n=10). *P<0.05 and **P<0.01 using one-way ANOVA with Dunnett’s test as a post hoc test.
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SH and 5HIAA activation of the AhR signaling in mouse colon or sera. a, c, e, Effect of SH treatment on mRNA levels of AhR (a) and its downstream genes CYP1A2 (c) and CYP1B1 (e). b, d, f, The effect of 5-HIAA on mRNA levels of AhR (b) and its downstream genes CYP1A2 (d) and CYP1B1 (f). The data are the mean ± sem (n=10). *P<0.05 and ***P<0.001 using one-way ANOVA with Dunnett’s test as a post hoc test.
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