

# Carrimycin Ameliorates Dextran Sulfate Sodium-Induced Ulcerative Colitis in Mice by Modulating Gut Microbiota and Restoring Th17/Treg Balance

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## Research Article

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**Carrimycin Ameliorates Dextran Sulfate Sodium-Induced Ulcerative  
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Balance**

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Abstract

Objective: To investigate the therapeutic potential and underlying molecular mechanisms of carrimycin (CA) in a murine model of dextran sulfate sodium (DSS)-induced ulcerative colitis (UC).

Methods: C57BL/6 male mice were randomly assigned to control, model, and carrimycin-treated (10 mg/kg) groups. Experimental colitis was induced by oral administration of DSS. Disease progression was evaluated by monitoring body weight changes and calculating the Disease Activity Index (DAI). Histopathological alterations in colonic tissues were assessed via hematoxylin and eosin (H&E) staining. Furthermore, flow cytometry was utilized to characterize shifts in immune cell populations, and 16S rRNA microbial sequencing was performed to analyze the composition and diversity of the gut microbiota.

Results: CA intervention significantly attenuated DSS-induced clinical symptoms, manifested by reduced body weight loss, lowered DAI scores, alleviated colon shortening, and decreased splenomegaly. Histopathological examination confirmed that CA effectively preserved colonic mucosal integrity and suppressed inflammatory cell infiltration. Systemically, CA significantly decreased the proportions of CD3<sup>+</sup> T cells, Gr-1<sup>+</sup> neutrophils, and circulating pro-inflammatory cytokines (IL-6, TNF- $\alpha$ , IFN- $\gamma$ ), while restoring B220<sup>+</sup> B cell populations. Notably, CA treatment restored the Th17/Treg balance in vivo by robustly downregulating Th17 cell frequencies and upregulating Treg cell populations in the colonic lamina propria, a mechanism corroborated by the in vitro polarization assay showing direct inhibition of Th17 differentiation and promotion of Treg differentiation in a dose-dependent manner. Additionally, 16S rRNA sequencing revealed that CA restored gut microbiota  $\alpha$ - and  $\beta$ -diversity, markedly reducing the relative abundance of pathogenic *Escherichia*-*Shigella* while enriching beneficial commensals such as *Akkermansia*.

Conclusion: These findings demonstrate that carrimycin effectively ameliorates DSS-induced ulcerative colitis by suppressing systemic inflammation, restoring colonic Th17/Treg homeostasis, and reconstructing the gut microecosystem, suggesting its therapeutic promise as an alternative immunomodulatory agent for UC management.

**Keywords:** Carrimycin; Gut microbiota; Ulcerative colitis; Inflammation

## Introduction

Ulcerative colitis (UC) is a chronic, idiopathic inflammatory bowel disease (IBD) primarily characterized by continuous mucosal inflammation originating in the rectum and extending

proximally through the colon. Clinically, UC presents with a spectrum of debilitating symptoms, including persistent diarrhea, hematochezia, abdominal cramping, and progressive weight loss. From a histopathological perspective, the disease is defined by diffuse acute and chronic inflammatory cell infiltration within the intestinal mucosa, often accompanied by crypt distortion and abscess formation. Over the past few decades, the global incidence and prevalence of UC have risen exponentially, expanding from historically high-incidence Western nations to rapidly industrializing regions in Asia, thereby imposing an escalating public health and economic burden worldwide. While the precise etiology of UC remains elusive, current consensus suggests a multifactorial pathogenesis involving a complex interplay between genetic predisposition, environmental triggers such as diet, gut dysbiosis, and dysregulated immune responses in susceptible hosts[1]. Mucosal immune dysregulation is a pivotal driver in the pathogenesis of ulcerative colitis (UC). The disease is hallmarked by a profound homeostatic imbalance within the intestinal immune compartment, specifically characterized by the exuberant production of pro-inflammatory Th1-type (TNF- $\alpha$ , IL-2, IL-12, and IFN- $\gamma$ ) and Th17-type (IL-17) cytokines, alongside a concomitant downregulation of anti-inflammatory Th2/regulatory cytokines (IL-10 and IL-4)[1, 2]. The mucosal immune system plays an indispensable role in maintaining intestinal epithelial homeostasis, wherein the adaptive helper T (Th) cell lineage balance is critically linked to mucosal integrity. Specifically, the dynamic equilibrium between pro-inflammatory Th17 cells and anti-inflammatory regulatory T (Treg) cells—collectively termed the Th17/Treg axis—functions as a pivotal immunological rheostat in the gut. Treg cells, characterized by the lineage-specific transcription factor Foxp3, promote immune tolerance and suppress aberrant inflammation through the secretion of regulatory cytokines such as IL-10 and TGF- $\beta$ . Conversely, Th17 cells, driven by the transcription factor ROR $\gamma$ t, secrete pro-inflammatory cytokines including IL-17A, IL-6, and TNF- $\alpha$ , which recruit neutrophils and exacerbate tissue injury. In patients with UC and corresponding animal models, this homeostasis is severely disrupted, shifting toward an exaggerated Th17 response and a functional deficiency or contraction of the Treg compartment. This immunological imbalance initiates a destructive cascade of mucosal inflammation, epithelial barrier breakdown, and tissue necrosis. Simultaneously, the intestinal microbiota serves as a foundational component of the intestinal defense system, participating actively in metabolic processes, barrier reinforcement, and immune education. Intestinal dysbiosis in UC is typically characterized by a marked reduction in microbial alpha diversity, a depletion of beneficial obligate anaerobes, and a

compensatory expansion of facultative pathogenic microbes, such as the genus *Escherichia-Shigella*. This microbial shift deprives the host of protective metabolites like short-chain fatty acids (SCFAs), while concurrently increasing luminal lipopolysaccharides (LPS), which penetrate the compromised epithelial barrier to perpetually stimulate mucosal immune cells via Toll-like receptors, establishing a vicious cycle of dysbiosis and inflammation. Consequently, therapeutic strategies capable of simultaneously reconstructing the gut microbiota architecture and restoring the systemic or local Th17/Treg balance hold immense clinical potential.

Current clinical management of UC primarily relies on 5-aminosalicylates(5-ASAs),glucocorticoids,immunomodulators(such as azathioprine and 6-mercaptopurine),biological agents,and Janus kinase(JAK)inhibitors.While these pharmacological interventions—particularly corticosteroids and targeted small-molecule drugs—are cornerstone therapies,their long-term clinical utility is frequently hampered by substantial economic burdens and a concerning safety profile,including the risks of opportunistic infections and potential malignancies[3].Consequently,there remains a critical unmet medical need for the development of novel,safe,and cost-effective oral small-molecule therapeutics to improve the long-term prognosis of UC patients.

In this context,carrimycin(CA),a novel macrolide antibiotic with potential immunomodulatory properties,has emerged as a promising candidate.Approved as a Category 1 innovative drug in June 2019,CA has successfully demonstrated a favorable safety and tolerability profile in Phase III clinical trials.It exhibits low oral dosing requirements,a low incidence of adverse reactions,broad antimicrobial activity,and a low probability of secondary resistance[4].Beyond its potent antimicrobial efficacy,emerging evidence suggests that CA possesses significant immunomodulatory capabilities.Specifically,CA has been shown to upregulate the expression of HLA-DR and expand CD8<sup>+</sup>T cell populations,while concurrently suppressing the production of key pro-inflammatory cytokines,including IL-6 and TNF- $\alpha$ [5].From a pharmacokinetic perspective,CA reaches its peak concentration in the murine intestinal tract within 0.5 hours post-administration.Notably,this rapid intestinal accumulation is associated with a significant reduction in IL-1 $\beta$  levels within small intestinal tissues[6].These findings underscore the pharmacological feasibility of utilizing CA to target intestinal inflammation and suggest its potential in restoring immune homeostasis within the gut microenvironment.

Our preliminary laboratory findings demonstrated that oral administration of CA significantly extended the survival of mice with lipopolysaccharide(LPS)-induced pneumonia, suggesting that its anti-inflammatory efficacy is independent of its direct antimicrobial properties. Furthermore, recent studies have reported that in murine sepsis models induced by LPS and cecal ligation and puncture(CLP), CA intervention enhances gut microbiota diversity, reduces the abundance of pathogenic bacteria, and promotes the proliferation of beneficial probiotics. These microbial shifts appear to alleviate systemic inflammation by modulating short-chain fatty acid(SCFA) metabolic pathways[7]. Mechanistically, CA has been shown to significantly inhibit the phosphorylation of TNF- $\alpha$  and the nuclear translocation of NF- $\kappa$ B-p65 in macrophages, thereby downregulating the expression of key pro-inflammatory mediators, including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6[7]. Given that the hallmark pathological features of ulcerative colitis(UC) involve intestinal immune dysregulation and gut dysbiosis—particularly the overproduction of IL-1 $\beta$  and TNF- $\alpha$ , which are critical drivers of mucosal ulceration—the reported inhibitory effects of CA on these cytokines suggest its potential therapeutic value for UC. However, whether carrimycin can effectively attenuate DSS-induced ulcerative colitis by modulating the gut microbiota and immune cell balance remains to be elucidated. Therefore, the present study was designed to evaluate the therapeutic effects of CA in a DSS-induced mouse model and to explore its potential mechanisms involving immune regulation and microbiota homeostasis. This research aims to provide a scientific rationale and expand clinical treatment options for the management of UC.

## Material and Methods

### 1. Experimental Animals

Male SPF C57BL/6 mice (6–8 weeks old, initial weight 20–22 g) were purchased from Beijing Charles River Laboratories. All mice were acclimatized for one week under Specific Pathogen-Free (SPF) conditions and reached an average weight of approximately 25 g prior to the commencement of the experiments. Animal husbandry and experimental procedures were conducted in strict accordance with the approved institutional guidelines (Animal Facility License: SYXK(Sichuan)2020-224; Ethics Approval No.: eB-D2024015). At the experimental endpoint, mice were deeply anesthetized with isoflurane (3–5% in oxygen) prior to blood collection. Peripheral blood was collected via the retro-orbital venous plexus for serum preparation and flow cytometric analysis following red blood cell lysis. Subsequently, mice were euthanized by cervical dislocation while under deep anesthesia. Death was confirmed by the absence of respiration and heartbeat before

tissue collection. All procedures were performed by trained personnel and were designed to minimize animal pain and distress.

## 2.Reagents and Antibodies

Dextran sulfate sodium(DSS)(M.W.36,000-50,000Da)was obtained from MP Biomedicals(USA).The test drug,carrimycin(CA),was provided by Shanghai Tonglian Pharmaceutical Co.,Ltd.Cytokine concentrations were quantified using a Cytometric Bead Array(CBA)kit(Biosciences,Cat#560485).The following fluorochrome-conjugated antibodies were utilized for flow cytometric analysis:BV421-anti-mouse-CD45R(B220)(Cat#48-0452-82,Invitrogen),APC-anti-mouse-Gr-1(Cat#108412,BioLegend),PerCP-anti-mouse-CD3e(Cat#553067,Biosciences),FITC-anti-mouse-CD4(Cat#100510,BioLegend),BV421--anti-mouse-CD25(Cat#562606,Biosciences),PE-anti-mouse-Foxp3(Cat#563101,BD Biosciences),and PerCP-Cy5.5-anti-mouse-IL-17A(Cat#45-7177-82,Invitrogen).

## 3.DSS-induced colitis and carrimycin administration

Mice(n=7/group)were randomized into Control,DSS model,and Carrimycin groups.Ulcerative colitis was induced in the latter two groups by providing 2.5%DSS in drinking water(replaced daily)from days 4 to 10.The Carrimycin group received carrimycin(10mg/kg in 30%cyclodextrin)daily via oral gavage starting on Day 4.Control and model mice received an equivalent volume of 30%cyclodextrin as a vehicle control.

## 4.Data Collection

### 4.1Disease Activity Index(DAI)Scoring

The clinical status of the mice was monitored daily,including changes in body weight,mental state,and hair luster.Stool consistency was categorized as normal,loose,or diarrhea,and the presence of gross or occult blood in the feces was recorded.Furthermore,the perianal area was inspected for fecal/blood residue,erythema,and edema.The severity of colitis was quantified using the Disease Activity Index(DAI),calculated as the sum of scores for weight loss,stool consistency,fecal occult blood,and fur condition,with a cumulative maximum score of 16 points.(Table1)

### **Table 1** Disease Activity Index(DAI)

| General Condition                             | Weight Loss(%) |       |   | Fecal Consistency                                       |   | Rectal Bleeding |   |
|-----------------------------------------------|----------------|-------|---|---------------------------------------------------------|---|-----------------|---|
| Normal                                        | 0              | 0     | 0 | Normal                                                  | 0 | No bleeding     | 0 |
| Hair standing on end                          | 1              | 1-5   | 1 |                                                         |   |                 |   |
| Hair standing on end and generalized weakness | 2              | 6-10  | 2 | Loose                                                   | 2 | Minor bleeding  | 2 |
|                                               |                | 11-15 | 3 |                                                         |   |                 |   |
| Immobility or weakness                        | 4              | >15   | 4 | Loose stools,or no defecation observed within 5 minutes | 4 | Blood in stool  | 4 |

183 Stool consistency was scored according to the following criteria:

184 (1)Normal:well-formed and firm pellets;

185 (2)Loose stool:paste-like or semi-formed pellets that did not adhere to the anus;

186 (3)Watery stool:liquid or unformed stool that adhered to the perianal area.

#### 187 4.2Measurement of Spleen Index

188 On day 10 of the experiment,the final body weight of each mouse was  
 189 recorded.Following euthanasia,the spleen was harvested,rinsed with ice-cold PBS to  
 190 remove residual blood,and gently blotted dry with filter paper.The spleen weight was then  
 191 accurately measured.The spleen index was calculated according to the following Spleen  
 192 Index=(Spleen Weight/Body Weight)×100%

#### 193 5.Histopathology

194 Colon tissues were fixed in 4%paraformaldehyde,embedded in paraffin,and sectioned  
 195 for hematoxylin and eosin(H&E)staining.Histopathological alterations were assessed via  
 196 light microscopy.The evaluation focused on key parameters including the maintenance of  
 197 structural integrity,glandular arrangement(crypt architecture),inflammatory cell  
 198 infiltration,depth of ulceration,extent of lesions,and the presence of mucosal congestion or  
 199 hemorrhage.

#### 200 **Table 2** Pathological Scoring Criteria

| Inflammation | Degree of lesion |                                   | Destruction of crypts |                                                | Extent of lesion (%) |        |   |
|--------------|------------------|-----------------------------------|-----------------------|------------------------------------------------|----------------------|--------|---|
| No           | 0                | No                                | 0                     | No                                             | 0                    | 1-25   | 0 |
| Mild         | 1                | Layer of mucous membrane          | 1                     | The basal 1/3 crypts were destroyed            | 1                    | 26-50  | 1 |
| Moderate     | 2                | The mucosal and submucosal layers | 2                     | The basal 2/3 crypts were destroyed            | 2                    | 51-75  | 2 |
| Severe       | 3                | Muscular layer                    | 3                     | Only the intact surface epithelium was present | 3                    | 76-100 | 3 |
|              |                  |                                   |                       | All crypts and epithelium were destroyed       | 4                    |        |   |

## 201 6.Serum Inflammatory Cytokine Assay

202 Following the experimental period,serum samples were harvested from all groups and  
203 stored at-80°C until analysis.The concentrations of key cytokines,including IL-2,IL-4,IFN-  
204  $\gamma$ ,TNF- $\alpha$ ,IL-6,IL-10,and IL-17A,were simultaneously quantified using a Cytometric Bead  
205 Array(CBA)kit(Biosciences San Jose,CA,USA)according to the manufacturer's  
206 instructions.Data were acquired via a flow cytometer(e.g.,BD FACSCanto II)and analyzed  
207 using the BD FCAP Array™software(version 3.0)to determine the absolute concentrations  
208 based on standard curves.

## 209 7.Immunophenotyping and Flow Cytometry Analysis

210 Peripheral blood 50 $\mu$ L was collected from mice,and erythrocytes were removed using  
211 RBC lysis buffer.For colonic mucosal cells,the mucosal layer was directly scraped and  
212 filtered through a 70 $\mu$ M cell strainer to obtain a single-cell suspension.To isolate lamina  
213 propria mononuclear cells(LPMCs),colons were opened longitudinally,rinsed of fecal  
214 matter,and incubated in EDTA-DTT buffer(37°C,250 rpm,15 min)to remove epithelial  
215 cells.The remaining tissue was minced and digested in RPMI 1640 medium containing 2  
216 mg/mL Collagenase IV and 50 $\mu$ g/mL DNase I at 37°C for 50 min.LPMCs were then  
217 recovered using a lymphocyte separation medium via density gradient  
218 centrifugation(400g,5min).Splenic single-cell suspensions were prepared by dispersing the  
219 tissue through a 70 $\mu$ M strainer followed by RBC lysis.

For general immune profiling, cells from blood and colonic mucosa were stained with a fixable viability dye and fluorochrome-conjugated antibodies against CD45, CD3e, B220, and Gr-1 at 4°C for 20 min in the dark. For Treg analysis (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>), cells were surface-stained with anti-CD4 and anti-CD25, followed by fixation and permeabilization using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set. Intracellular staining for Foxp3 (clone FJK-16S) was performed according to the manufacturer's instructions. For Th17 analysis, LPMCs were stimulated with PMA (50 ng/mL), ionomycin (1 µg/mL), and monensin (3 µM) in RPMI 1640 medium for 4 h at 37°C. Subsequently, cells were stained for surface CD4 and intracellular IL-17A using the BD Fixation/Permeabilization kit.

Naïve CD4<sup>+</sup>T cells were purified from splenic suspensions using CD4(L3T4) MicroBeads via magnetic-activated cell sorting (MACS). Purified cells were seeded into culture plates with lineage-specific differentiation cocktails and treated with Carrimycin at concentrations of 0, 0.5, and 1 µM. On day 3, fresh medium containing cytokines and Carrimycin was supplemented (total volume 300 µL/well) without medium replacement to maintain the integrity of lymphocyte clusters. On day 5, the proportions of Th17 and Treg subsets were quantified. All data were acquired using a flow cytometer and analyzed with FlowJo V10 software.

#### 8.16S rRNA microbial sequencing

Total genomic DNA was extracted from fecal samples by Suzhou Genevix Biotechnology Co., Ltd. (Suzhou, China). The V3–V4 hypervariable regions of the 16S rRNA gene were amplified using specific primers. Subsequently, indexed adapters were attached to the PCR products for next-generation sequencing (NGS). The quality and size of the PCR libraries were verified using 1.5% agarose gel electrophoresis to confirm target fragments of approximately 600 bp. Paired-end sequencing (PE250/PE300) was performed on the Illumina MiSeq/NovaSeq platform (Illumina, San Diego, CA, USA) to generate raw sequence data.

Raw reads were merged and quality-filtered to remove sequences containing ambiguous bases or those shorter than 200 bp. Following chimera removal, high-quality sequences were clustered into Operational Taxonomic Units (OTUs) with a 97% similarity threshold using VSEARCH (v1.9.6). Taxonomic assignment was performed against the Silva 138 reference database using the RDP classifier (Ribosomal Database Program) with a Bayesian algorithm.

To evaluate community composition,  $\alpha$ -diversity indices, including Shannon and Chao1, were calculated to assess species richness and evenness. Rank-abundance curves were plotted to visualize the distribution of species. To compare microbial community structures across groups,  $\beta$ -diversity was visualized using Principal Component Analysis (PCA), Principal Coordinate Analysis (PCoA), and Non-metric Multidimensional Scaling (NMDS) based on Bray-Curtis or UniFrac distances.

## 9. Statistical analysis

Statistical analysis and graphical representation were performed using GraphPad Prism software (version 9.0; GraphPad Software, San Diego, CA, USA). Data are presented as the mean  $\pm$  standard error of the mean (SEM). For comparisons among multiple groups, a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was employed. Differences between two groups were evaluated using the independent-samples t-test. Statistical significance was defined at  $P < 0.05$ .

## Results

### Carrimycin Attenuates Clinical Signs and Symptoms in DSS-Induced Colitis

During the acclimation period, all mice exhibited normal clinical status and regular defecation. Following acclimation, mice were randomly assigned to the control group, DSS group, or Carrimycin-treated group. Starting from Day 1, the control group received normal drinking water, while the DSS and Carrimycin groups were administered 2.5% DSS in their drinking water for 7 consecutive days (with fresh DSS solution replaced daily). Concurrently, the Carrimycin group received a daily oral dose of 10 mg/kg Carrimycin. Throughout the experiment, the control group maintained stable body weight, active behavior, and normal fur condition. In contrast, mice in the DSS model and Carrimycin groups began to exhibit weight loss on Day 4, accompanied by lethargy and ruffled fur. Significant weight loss was observed between Days 5 and 7 (Fig. 1b), along with clinical signs such as fecal adhesion in the perianal area. The Disease Activity Index (DAI) was employed to assess colitis severity (Fig. 1c). While the control group maintained a DAI score of 0, the model group showed a significant upward trend in DAI scores as inflammation progressed, confirming the successful establishment of the colitis model. Notably, Carrimycin treatment significantly alleviated these clinical symptoms. On Days 7 and 9, the Carrimycin group exhibited significantly lower DAI scores compared to the model group ( $P < 0.001$ ), demonstrating its efficacy in inhibiting the progression of DSS-

283 induced colitis. Additionally, Carrimycin partially mitigated the DSS-induced reduction in  
284 body weight (Fig. 1b).

285 The spleen index and colon length were measured as macroscopic indicators of colitis  
286 severity (Fig. 1d and Fig. 1e). Compared to the control group, the model group exhibited signifi-  
287 cant splenomegaly ( $P < 0.001$ ) and marked colon shortening ( $P < 0.001$ ), confirming severe syst-  
288 emic inflammation and intestinal tissue damage. Following Carrimycin (10 mg/kg) interventio-  
289 n, a trend toward improvement was observed, characterized by a decreased spleen index and  
290 partial recovery of colon length. However, statistical analysis revealed that these differenc-  
291 es did not reach significance ( $P > 0.05$ ). This suggests that under the current experimental co-  
292 nditions, a 10 mg/kg dose of Carrimycin may be insufficient to fully reverse the macroscopic  
293 tissue damage induced by acute colitis.

294 H&E staining was performed to evaluate the microscopic alterations in the colon (Fig. 1  
295 f). The control group displayed well-defined histological layers, including distinct mucosa, su-  
296 bmucosa, and muscularis, with regularly arranged intestinal glands and intact crypt structur-  
297 es (Histopathology score = 0). Conversely, the DSS-treated model group exhibited mucosal ed-  
298 ema, thickening of the lamina propria and muscularis, and extensive crypt loss or distortion.  
299 Ulcerative lesions were observed, characterized by robust inflammatory cell infiltration, exte-  
300 nsive goblet cell degeneration, and intestinal hemorrhage. Consequently, the pathological sc-  
301 ore of the model group ( $6.9 \pm 0.3$ ) was significantly higher than that of the control group ( $P < 0.0$   
302  $01$ ). The Carrimycin-treated group showed modest improvements in mucosal edema and tis-  
303 sue thickening. Compared to the model group, this group exhibited reduced inflammatory c-  
304 ell infiltration and better-preserved crypt architecture with a more regular arrangement. The  
305 resulting pathological score ( $4.5 \pm 0.4$ ) was significantly lower than that of the model group ( $P$   
306  $< 0.05$ ), confirming the protective effect of Carrimycin on the colonic mucosal barrier.

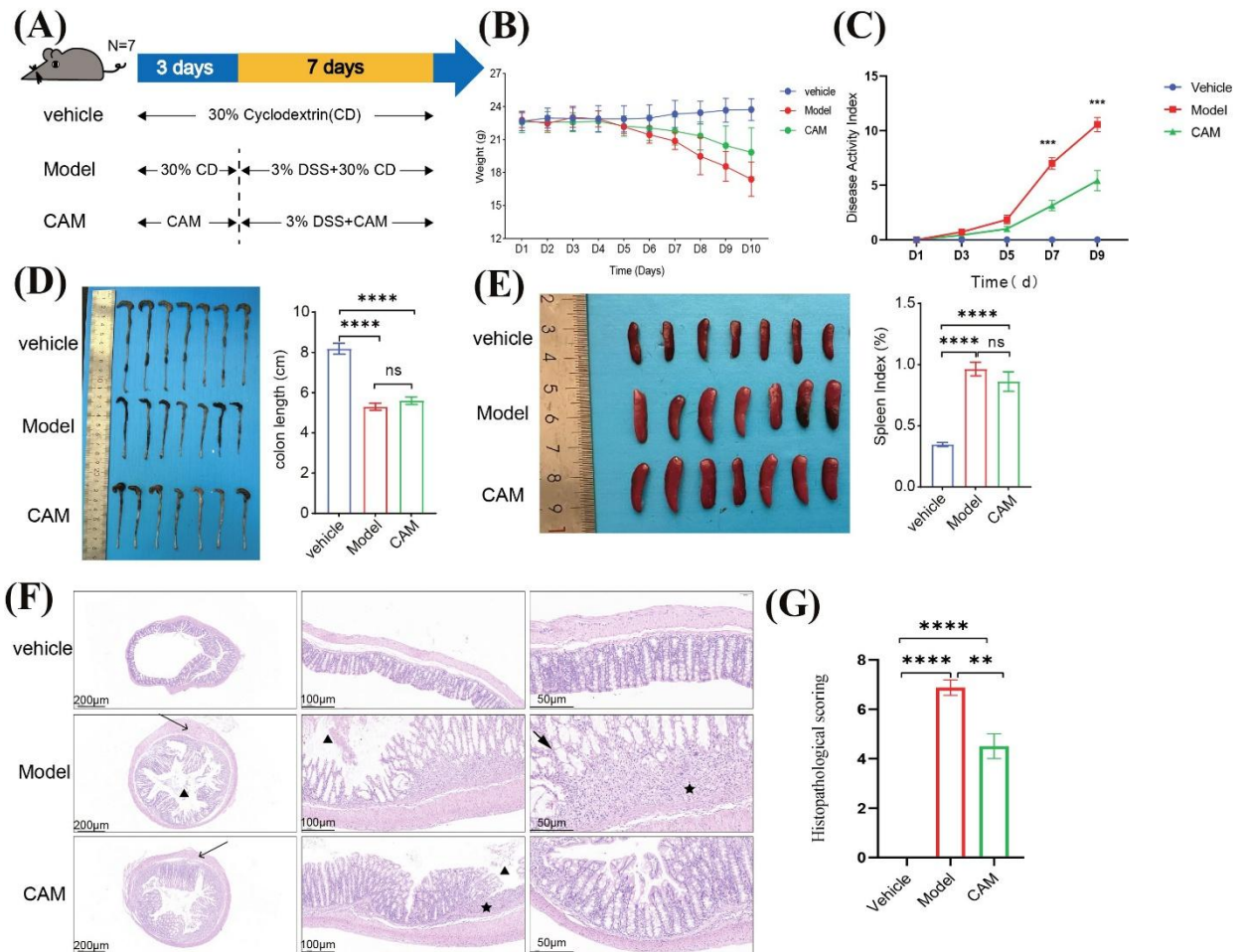


**Fig. 1** Carrimycin attenuates the severity of DSS-induced ulcerative colitis in mice.

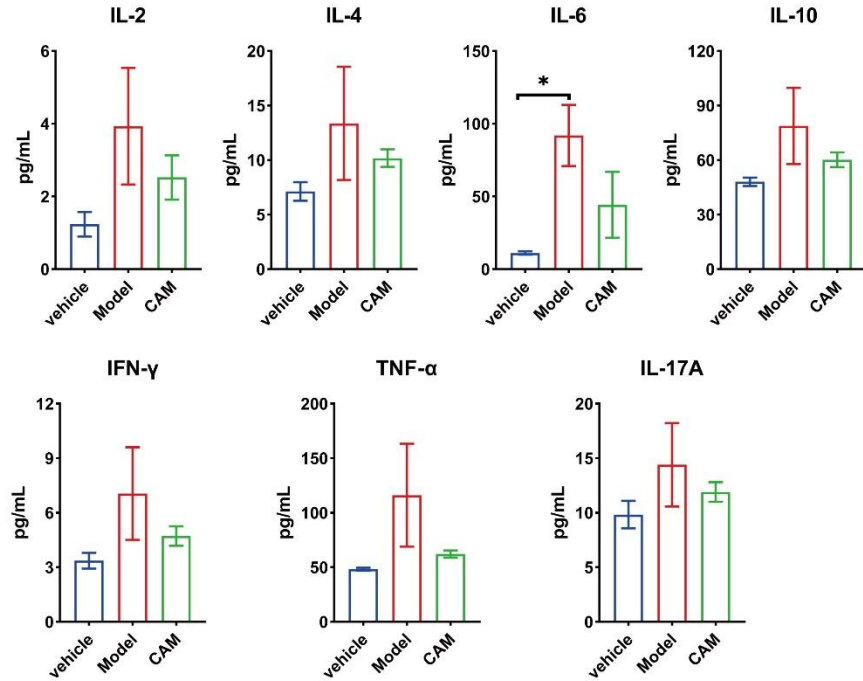
**A** Schematic representation of the experimental design, including the timeline for DSS induction and Carrimycin administration. **B** Dynamic changes in body weight over the 10 day experimental period (n=7 per group). **C** Disease Activity Index (DAI) scores recorded daily (n=7 per group).

er group). **D** Representative macroscopic images and statistical analysis of colon length( $n=7$ per group). **E** Spleen index( $n=7$ per group). **F** Representative HE-stained images of colonic sections(Scale bar= $200\mu\text{m}/100\mu\text{m}/50\mu\text{m}$ ).Pathological hallmarks are indicated as follows: extensive mucosal ulceration and hemorrhage( $\blacktriangle$ )cell infiltration( $\star$ );and submucosal edema with epithelial sloughing( $\uparrow$ ). **G** Histopathological scoring of colonic tissues based on H&E staining( $n=4$ per group).Data are presented as the Mean $\pm$ SEM.Statistical significance: \* $P<0.05$ , \*\* $P<0.01$ ,and \*\*\* $P<0.001$ .

### Carrimycin Suppresses Pro-inflammatory Cytokines and Modulates Peripheral Immune Profiles

To explore the anti-inflammatory mechanism of the drug,we measured the concentrations of key cytokines in the serum.The results(Fig.2a and Fig.2b)showed an upward trend in the expression of various pro-inflammatory cytokines in the model group.Notably,IL-6,a key pro-inflammatory cytokine,was significantly elevated in the model group( $P<0.05$ ),verifying the successful establishment of the model.Following Carrimycin( $10\text{mg/kg}$ )intervention,IL-6 levels decreased by approximately 52%,demonstrating a clear inhibitory effect.Concurrentlly,we observed highly consistent patterns in TNF- $\alpha$ ,IFN- $\gamma$ ,IL-2,and IL-17A levels:they were significantly upregulated in the model group,whereas the treatment group restored them to levels approximating the normal controls.Although individual variations among mice resulted in some differences not reaching statistical significance( $P>0.05$ ),the consistent downward trend across multiple indicators suggests that Carrimycin holds the potential to broadly suppress the storm of related pro-inflammatory cytokines.

(A)



(B)

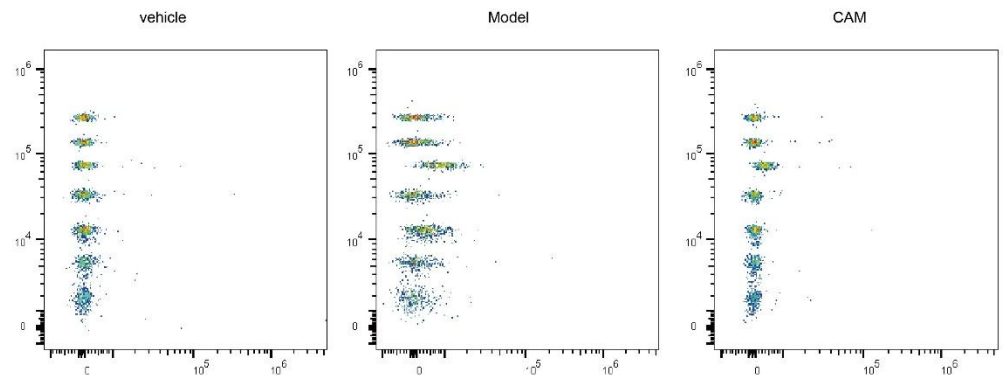


**Fig. 2** Effect of Carrimycin on Cytokine Expression Levels in DSS-Induced Colitis Mice.

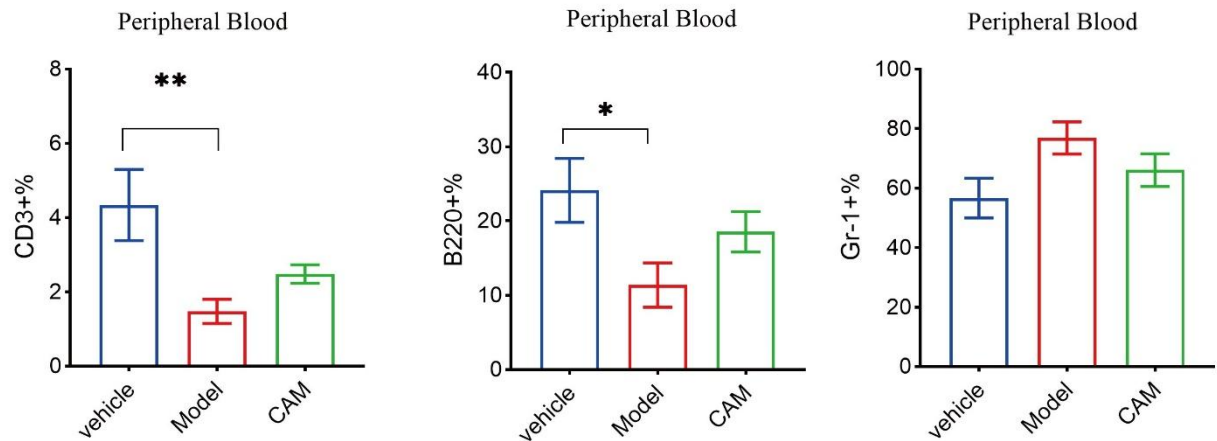
**A** Quantitative analysis of cytokines. **B** Representative flow cytometry scatter plots.

.Data are presented as the Mean±SEM.Statistical significance: \* $P < 0.05$

Flow cytometry analysis(Fig.3)revealed that colitis modeling triggered a systemic immune imbalance in mice.Compared to the control group,the proportion of CD3+T cells and B220+B cells in the peripheral blood of the model group was significantly decreased( $P < 0.05$ ),accompanied by an upward trend in the proportion of Gr-1+granulocytes( $P > 0.05$ ).Followi

341 ng Carrimycin treatment,the systemic immune status showed signs of recovery:the proport  
342 ion of CD3+T cells in the peripheral blood rebounded to around 2%,B220+cell proportions  
343 also recovered,and the abnormally elevated Gr-1+granulocyte levels declined.Although the  
344 se changes did not reach statistical significance( $P>0.05$ ),the trend of improvement was cle  
345 ar.In the local intestinal tissues,we observed an immunomodulatory pattern highly consiste  
346 nt with the peripheral blood results.The proportion of Gr-1+cells in the intestinal tissues of t  
347 he model group increased approximately 7-fold,indicating massive neutrophil infiltration in  
348 to the inflammatory sites,which aligned with the intestinal pathological findings.While indiv  
349 idual differences within the Carrimycin treatment group led to non-significant statistical dif  
350 ferences( $P>0.05$ ),the numerical proportion of Gr-1+cells was substantially downregulated.I  
351 n summary,the consistent improvement trends in both local and systemic immune marker  
352 s within the treatment group suggest that Carrimycin exerts a potential regulatory role in all  
353 eviating colitis-associated systemic immune dysregulation.

(A)



(B)

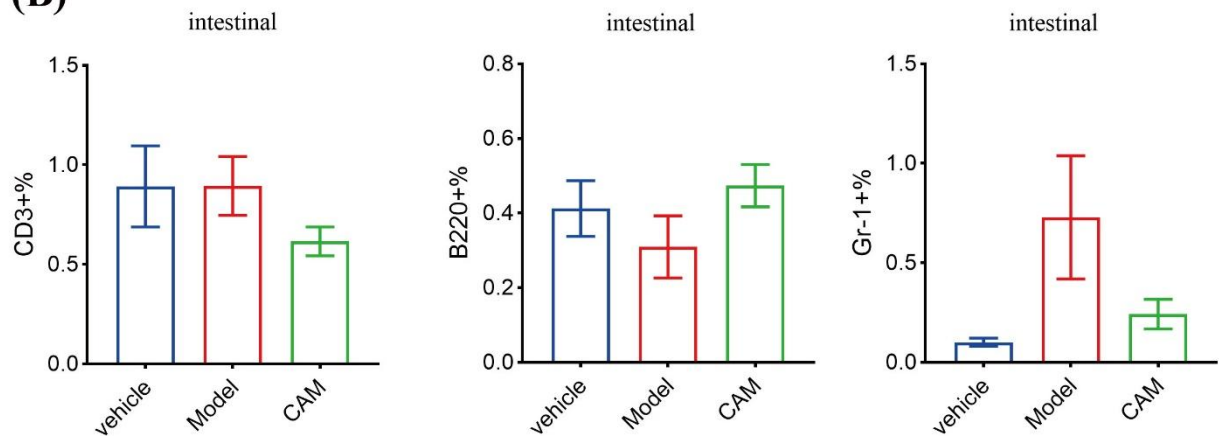
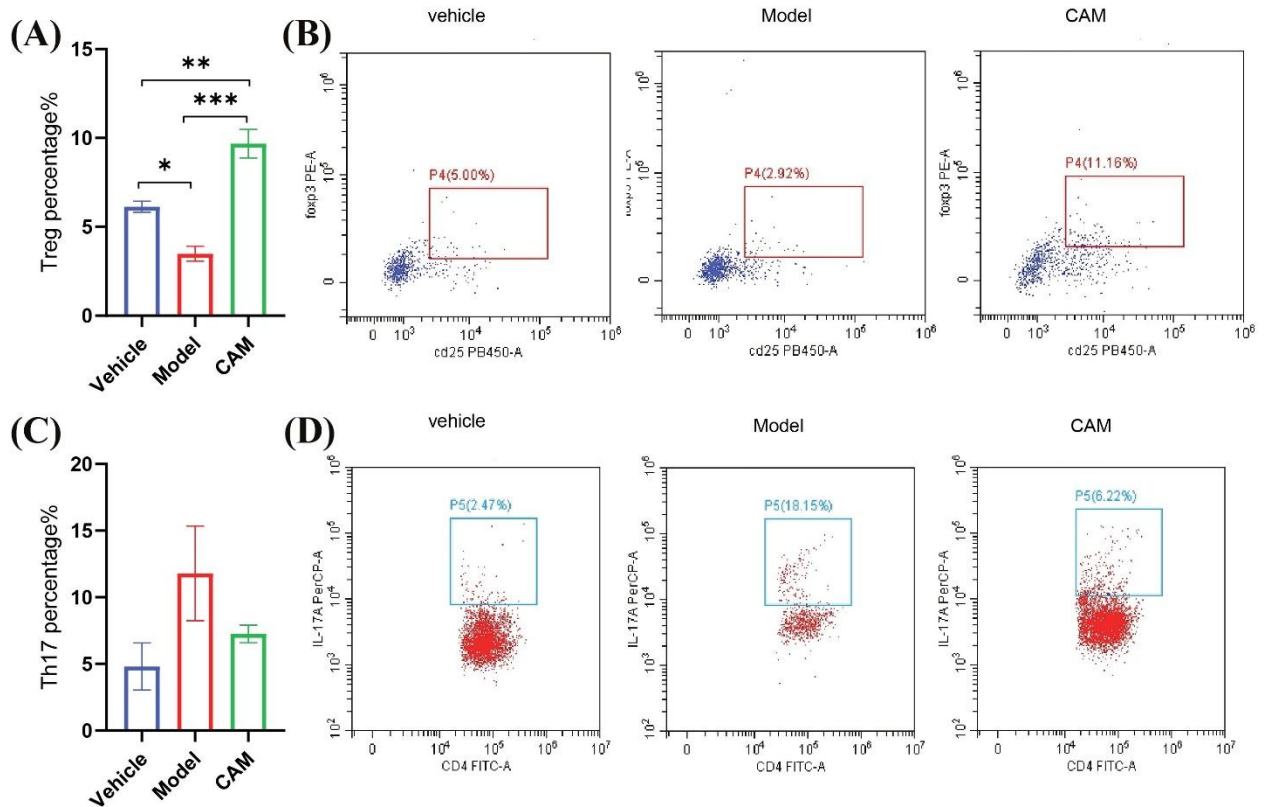


**Fig. 3** Effect of Carrimycin on Immune Cell Proportions in the Peripheral Blood and Intestine. Data are presented as the Mean±SEM. Statistical significance: \* $P<0.05$ , \*\* $P<0.01$ .

### Carrimycin Restores the Mucosal Th17/Treg Cell Balance In Vivo

Th17 cells mediate inflammatory responses, while Treg cells mediate immune tolerance and homeostasis. Th17 and Treg cells are typically in dynamic equilibrium within the body; an imbalance between them promotes the occurrence and development of various inflammatory diseases by impairing systemic immunity and anti-inflammatory capacity. We detected the distribution of Th17 and Treg cells in the colonic lamina propria across all groups using flow cytometry. Compared with the control group, the proportion of CD4+CD25+Foxp3+ Treg cells, which possess immunosuppressive functions, was significantly decreased in the colonic lamina propria of the model group. Following Carrimycin intervention, the proportion

of Treg cells rebounded significantly, demonstrating a potent immune-inducing and reparative effect. The mean proportion of CD4+IL-17A+Th17 cells in the intestines of the model group was notably higher than that of the control group. After Carrimycin administration, this mean proportion decreased. Although large individual variances in the model group prevented this downregulation trend from reaching statistical significance ( $P>0.05$ ), the numerical shift is consistent with the overall anti-inflammatory phenotype.



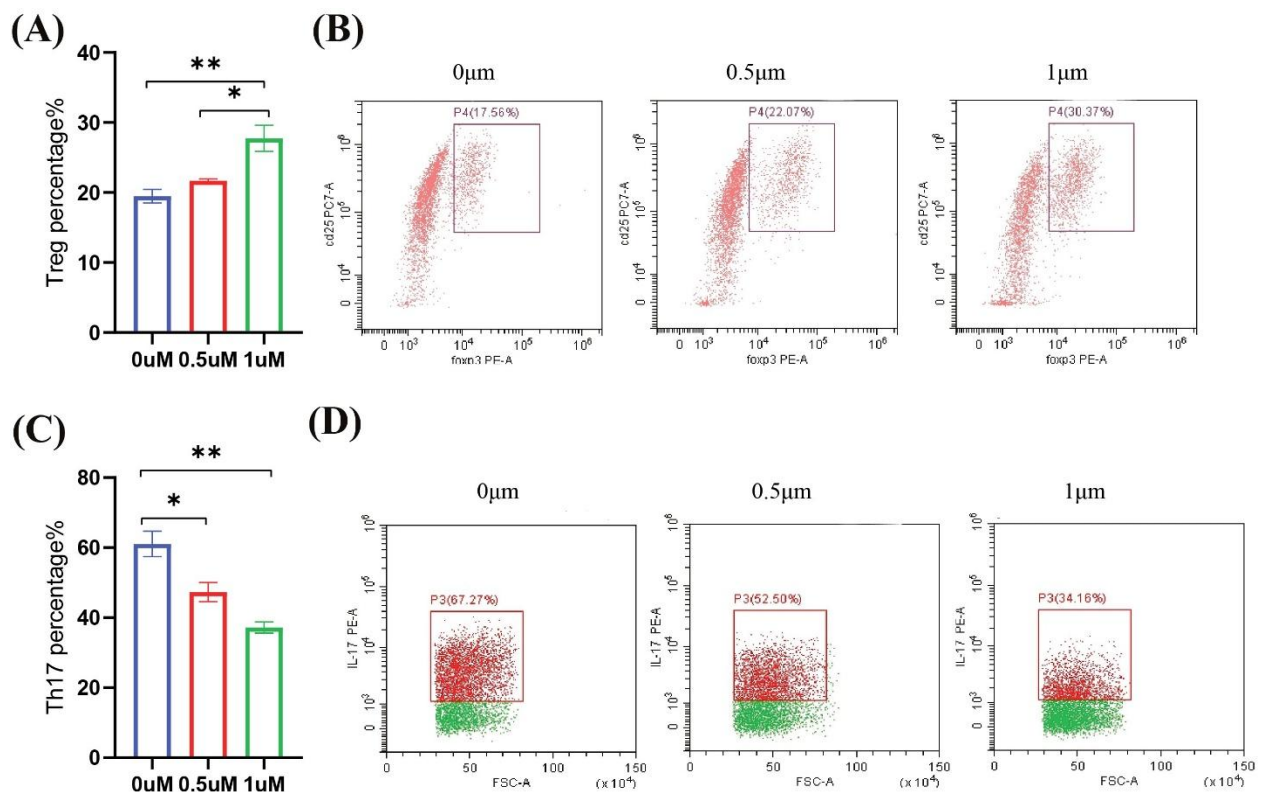
372

373 **Fig. 4** Carrimycin Regulates the Th17/Treg Immune Balance in the Colonic Lamina Propria  
374 of Colitis Mice.

375 **A** Statistical analysis of Treg cell proportions in the intestinal lamina propria. **B**  
376 representative flow cytometry scatter plots of Treg cells (Gate: CD4+CD25+Foxp3+). **C**  
377 Statistical analysis of Th17 cell proportions in the intestinal lamina propria. **D**  
378 representative flow cytometry scatter plots of Th17 cells (Gate: CD4+IL-17A+). Data are  
379 expressed as Mean±SEM, n=3, Statistical significance: \* $P<0.05$ , \*\* $P<0.01$ ; the differences in  
380 Th17 proportions among groups were not statistically significant ( $P>0.05$ ).

381 Carrimycin Promotes T Cell Differentiation into Treg Cells

382 We designed in vitro experiments to verify the pro-differentiative effects of Carrimycin  
383 on T cells. Mouse splenocytes were isolated, and T cells were induced to differentiate  
384 toward the Th17 or Treg lineages under appropriate cytokine concentrations. During the cell  
385 culture process, the addition of different concentrations of  
386 Carrimycin (0  $\mu$ M, 0.5  $\mu$ M, 1  $\mu$ M) resulted in a significant, dose-dependent inhibition of Th17  
387 cell generation. This result indicates that Carrimycin can directly block the formation of pro-  
388 inflammatory T cells. Conversely, in the Treg induction system, the addition of Carrimycin  
389 significantly elevated the proportion of CD4+CD25+Foxp3+Treg cells, a finding congruent  
390 with the in vivo observations of colonic innate immune cells.



391  
392 **Fig. 5** Carrimycin Directionally Regulates the Differentiation of Mouse CD4+T Cells into  
393 Treg/Th17 Lineages In Vitro.

394 **A** Statistical histogram of Carrimycin promoting Treg differentiation. **B** Representative flow  
395 cytometry scatter plots of in vitro induced CD4+CD25+Foxp3+Treg cells under varying

Carrimycin concentrations(0μM,0.5μM,1μM). **C** Statistical histogram of Carrimycin inhibiting Th17 differentiation. **D** Representative flow cytometry scatter plots of in vitro induced CD4+IL-17A+Th17 cells under varying Carrimycin concentrations(0μM,0.5μM,1μM). Data are expressed as Mean±SD,n=3,Statistical significance:\* $P<0.05$ ,\*\* $P<0.01$ (compared with the 0μM group).

#### Carrimycin Reconstructs the Diversity and Structural Composition of the Gut Microbiota

High-throughput16S rRNA sequencing was performed on the colonic microbiota of mice to obtain OTU clusters,allowing for the assessment of microbial community diversity.As illustrated in the Venn diagram,the control,model,and Carrimycin groups shared 40 OTUs;the control group had 161 unique OTUs,the model group had 1 unique OTU,and the Carrimycin group had 45 unique OTUs.The Rank-Abundance curve(Fig.6b)further delineated the richness and evenness of the communities in each group.The control group's curve exhibited the widest span and a gentle slope,indicating the highest species richness and evenness.The model group's curve span on the horizontal axis was drastically shortened,with a steeper slope,reflecting a severe loss of species richness and profound community unevenness induced by the modeling.Following Carrimycin intervention,the curve's coverage expanded slightly,indicating a partial restoration of the community's ecological breadth.These results demonstrate that DSS significantly impacts the gut microbiota of mice,and that Carrimycin can partially modulate the structural alterations in the gut microbiota of DSS-induced UC mice.

Beta diversity was evaluated via distance-based PCoA.The results(Fig.6c)showed that the first principal coordinate(PC1)explained 65%of the community variation.The control and model groups were clearly separated along the PC1 axis,and this separation was statistically significant( $P<0.05$ ).Post-intervention,the samples from the Carrimycin group migrated significantly toward the control group in the spatial distribution,indicating that the drug effectively regulated the community structure under pathological conditions,driving it back toward a normal physiological state.Alpha diversity analysis(Fig.6d)revealed highly significant differences in species richness and diversity across the groups( $P<0.001$ ).Compared to the control group,the model group exhibited significantly reduced Chao1 and Shannon indices,confirming microbial community atrophy due to the pathological state.Although the Carrimycin group did not show a pronounced recovery in total richness,its distinct community evolution pattern warrants further parsing via species abundance analysis.Overall,the results indicate that DSS induction significantly degrades

the species diversity of the mouse gut microbiota, while Carrimycin intervention can effectively ameliorate it.

In the genus-level taxonomic composition analysis (Fig. 6e), the gut microbiota of the model group exhibited distinct dysbiosis, characterized by a significant increase in the relative abundance of the pathogenic *Escherichia-Shigella* genus, alongside a drastic decline in the proportion of commensal bacteria such as *Lactobacillus*. Carrimycin intervention induced a specific remodeling of the community structure, with the most notable change being a sharp elevation in the abundance of *Akkermansia*, a bacterium recognized as a critical driver of intestinal barrier protection. Furthermore, the recovery of *Alistipes* and *Alloprevotella* in the treatment group suggests that Carrimycin may exert its therapeutic potential by enhancing short-chain fatty acid (SCFA) metabolism.

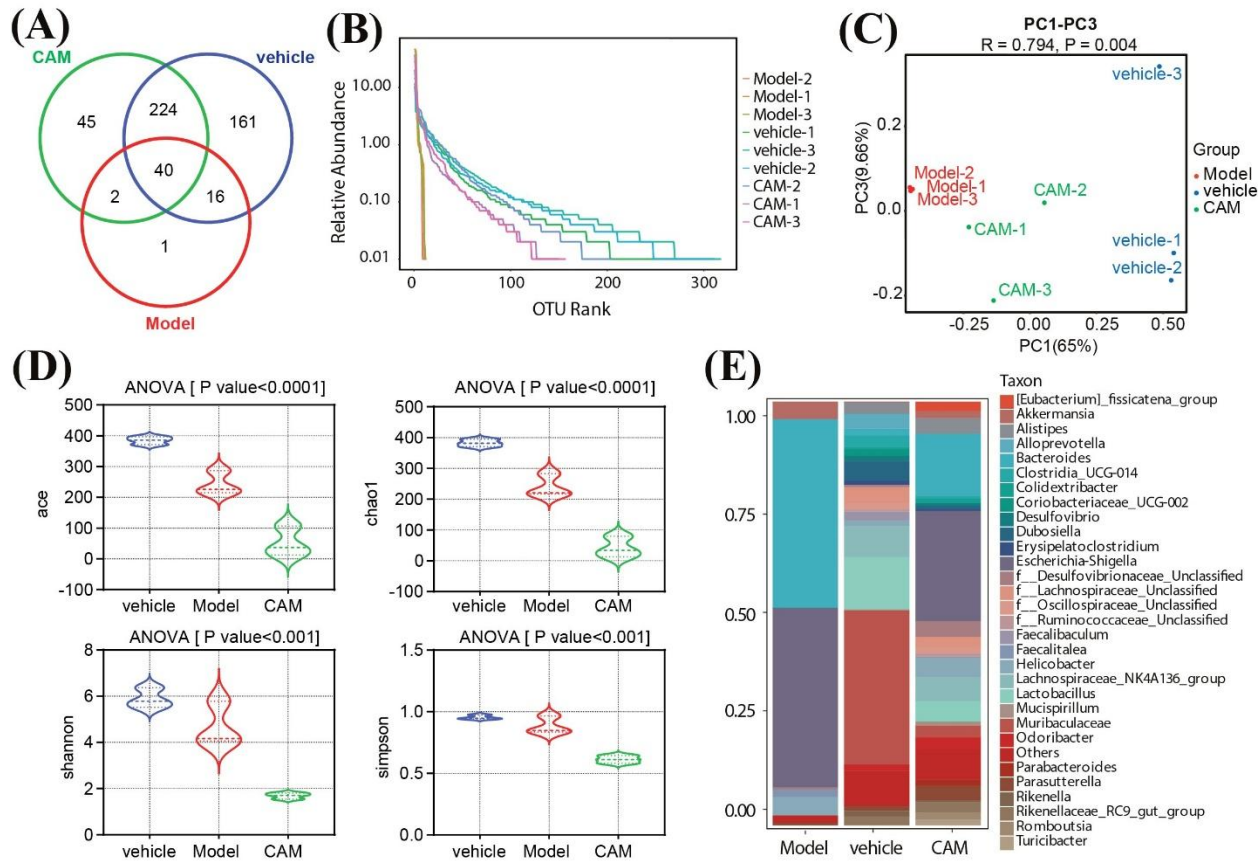


**Fig. 6** Effect of Carrimycin on the Structure and Diversity of Mouse Gut Microbiota.

**A** Venn diagram. **B** Rank-Abundance curve. **C** PCoA results. **D** Alpha diversity index analysis among groups. **E** Species abundance histogram.

## Discussion

The pathogenesis of Ulcerative Colitis(UC) involves impaired intestinal physical barrier s, mucosal immune dysregulation, and intestinal microecological disorders. Although current clinical treatments predominantly utilize steroids, aminosalicylates, immunosuppressants, and biologics, long-term administration often leads to adverse reactions and side effects; moreover, the complexity of the pathogenic factors frequently limits the efficacy of monotherapies[8, 9]. Carrimycin(CA), a novel macrolide antibiotic independently developed in China, was initially approved for clinical use due to its broad-spectrum antibacterial activity. With continuous research revealing its antibacterial, anti-inflammatory, and safety profiles, it has gained recognition from the NMPA[7]. Previous studies have validated CA's anti-inflammatory potential in various pathological models. For instance, a multicenter, double-blind, randomized controlled trial targeting tumor patients with sepsis demonstrated that CA significantly improved patients' inflammatory biomarker levels and immune functions[5]. In hepatocellular carcinoma research, CA exhibited not only anti-inflammatory effects but also potential anti-tumor capabilities via multi-target interventions[6]. Therefore, we investigated the therapeutic efficacy of Carrimycin on ulcerative colitis and its underlying mechanisms. In this study, the model group mice presented with typical weight loss, hematochezia, and significantly elevated DAI scores; Carrimycin intervention significantly reduced these DAI scores ( $P < 0.001$ ) and stabilized body weight. H&E staining results indicated that in the DSS model group, colonic ulcers extended to the muscular layer, with missing crypts, irregular glandular arrangement, massive inflammatory cell infiltration, and capillary dilation with hyperemia. After Carrimycin intervention, the colonic histopathological score significantly dropped from  $6.9 \pm 0.3$  in the model group to  $4.5 \pm 0.4$  ( $P < 0.05$ ). The CA group exhibited a more intact mucosal structure and significantly reduced inflammatory cell exudation, confirming Carrimycin's protective role regarding the intestinal physical barrier.

Although CA demonstrated a recovery trend in improving colon length and the spleen index, it did not reach statistical significance ( $P > 0.05$ ). This outcome suggests that during the acute inflammatory phase, a 10 mg/kg dose of CA may prioritize the microscopic repair of mucosal structures and the blockade of local inflammatory signaling, whereas a longer intervention period may be required to achieve macroscopic morphological reversal of tissue organs. Concurrently, this study presents the experimental results of low-dose Carrimycin; administration of high doses in preliminary trials did not yield additional benefits, indicating that the dosage significantly influences outcomes and that high-dose interventions fail to pro

duce expected dose-dependent synergistic effects. This phenomenon reflects the distinct therapeutic window characteristic of macrolide drugs. Previous studies have confirmed that macrolide and polyene antibiotics exert complex, synergistic immune-enhancing effects at sub-inhibitory (non-antibacterial) doses. For example, low-dose amphotericin B utilizes the oxidative or non-oxidative mechanisms of macrophages to fortify the body's native anti-fungal immune defenses [10]. Research also indicates that antibacterial doses of macrolides, such as clarithromycin, can cause severe and long-term disruption to the gut microecology [11]. This non-antibacterial immunomodulation often possesses higher receptor affinity and bioavailability at low doses, whereas excessive doses may trigger severe secondary fluctuations in the microecology, offsetting the immune protection benefits. This dose-sensitivity profile provides a critical reference for the potential clinical application of Carrimycin in treating colitis.

H&E staining results demonstrated a reduction in inflammatory cell infiltration in the colons of the treated mice, suggesting that Carrimycin may halt disease progression by regulating the recruitment and secretion of local intestinal immune cells. Consequently, we further investigated its impact on systemic inflammatory factors and the proportions of specific immune cells. The results showed that serum IL-6 levels in the model group were significantly elevated, and CA intervention reduced these levels by approximately 52%. Simultaneously, key pro-inflammatory cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , and IL-17A exhibited a consistent downregulation trend in the treatment group. This aligns with the findings of Nan et al., who reported that Carrimycin significantly reduces IL-6 and TNF- $\alpha$  levels in a sepsis model [5, 7]. Research has established that the Th17/Treg imbalance plays a pivotal role in the progression of intestinal diseases; IL-17, IL-6, and other pro-inflammatory factors produced by Th17 cells can recruit large numbers of neutrophils and promote intestinal inflammation. Conversely, Treg cells produce anti-inflammatory factors like IL-10, which suppress the activity of various immune cells, thereby inhibiting intestinal immune responses and inflammation [5, 12-14]. IL-6 can induce the expression of the pro-inflammatory transcription factor ROR $\gamma$ t and block the transcriptional activation of Foxp3, leading to a Th17/Treg imbalance. By significantly downregulating IL-6, CA inhibited the over-activation of Th17 cells and promoted the recovery of the Foxp3<sup>+</sup>Treg cell proportion in the colonic lamina propria. The dose-dependent promotion of Treg generation by CA in our in vitro differentiation assays further excluded antibacterial interference, verifying its direct modulatory capacity on T cell immune homeostasis.

The elevated proportion of Gr-1+ cells in both the intestine and peripheral blood of the model group reflects massive recruitment of myeloid cells such as neutrophils; this is consistent with the massive inflammatory cell infiltration observed in the colonic tissues via H&E staining, suggesting the body was under intense inflammatory stress. The declined proportion of CD3+ T cells and B220+ B cells in the peripheral blood of the model group indicates compromised immune function. The restoration of the Gr-1+ proportion in the treatment group, coupled with the recovery trend of CD3+ T cells and B220+ B cells, demonstrates that CA can reconstruct intestinal immune homeostasis. This is likely closely tied to CA's capacity to drive T cell differentiation toward regulatory T cells, offsetting Th17-mediated excessive immune responses by enhancing endogenous immunosuppressive forces. A pivotal finding of the present study is the specific and powerful regulatory effect of CA on the Th17/Treg balance. In the colonic lamina propria, DSS colitis triggers a massive shift toward a pro-inflammatory Th17 profile at the expense of protective Foxp3+ Treg cells. CA treatment profoundly reversed this shift in vivo. To delineate the underlying mechanism, our in vitro naive CD4+ T cell differentiation assay demonstrated that CA directly inhibited Th17 polarization and promoted Treg differentiation in a clear dose-dependent manner. This confirms that CA possesses cell-autonomous, direct immunomodulatory properties. Pharmacologically, CA may interact directly with lineage-specific transcription factors or alter upstream signaling cascades, such as the STAT3/STAT5 pathways. STAT3 phosphorylation is mandatory for ROR $\gamma$ t upregulation and Th17 skewing, whereas STAT5 activation promotes Foxp3 expression and Treg commitment. Many immunomodulatory small molecules modulate this checkpoint, and carrimycin's direct actions on T-cell differentiation strongly parallel these mechanisms, a subject we aim to explore further in molecular detail.

Intestinal immune imbalance and gut microbiota dysbiosis are central to the onset and progression of Ulcerative Colitis (UC). Recent studies confirm that gut microbes play a pivotal role in the development and clinical outcomes of UC [15]. The 16S rRNA sequencing results revealed a drastic shift in the gut microbiota structure of the model group. PCoA analysis showed a complete separation between the model and control groups, while the Carrimycin group exhibited a unique clustering profile. In the model group, *Escherichia-Shigella* demonstrated explosive growth. These facultative anaerobes are frequently considered biomarkers of intestinal inflammation; the lipopolysaccharide (LPS) they produce is a critical stimulus triggering the release of pro-inflammatory factors like TNF- $\alpha$  and IL-6 [16]. The most striking

g finding of this study is that Carrimycin intervention almost completely eradicated the abundance of *Escherichia-Shigella* in the gut. This potent suppression of a specific pathogen blocks the pro-inflammatory signaling pathways mediated by dysbiosis, thereby alleviating intestinal inflammation at its source. Simultaneously, CA significantly enhanced the abundance of the probiotic *Akkermansia*. As a critical commensal bacterium maintaining intestinal homeostasis, *Akkermansia* and its metabolites—short-chain fatty acids (SCFAs)—play crucial roles in regulating epithelial permeability [17]. Bian et al. confirmed that *Akkermansia* can enhance the expression of colonic tight junction proteins by activating specific metabolic pathways [18]. This provides direct mechanistic support for the improved crypt structures observed in the treatment group of our study. Furthermore, the optimized microbiota environment downregulated the local recruitment of myeloid cells, resulting in a sharp decline in local intestinal Gr-1<sup>+</sup> cell infiltration.

This study concludes that CA exerts a dual preventative and therapeutic effect on colitis: its broad-spectrum antibacterial properties selectively clear pro-inflammatory pathogens—represented by *Escherichia-Shigella*—to remodel the gut microbial community. We observed that Carrimycin intervention significantly enriched *Akkermansia*. As an obligate mucin-degrading bacterium, *Akkermansia* degrades mucin to produce acetate and propionate; these metabolites not only provide energy for colonocytes but also bind to G-protein-coupled receptors (e.g., GPR41/43) to regulate host immune balance and maintain intestinal integrity. This finding corresponds with Lai et al.'s research, which stated that Carrimycin alleviates inflammation by enhancing SCFA metabolic pathways [7]. SCFAs like butyrate serve as endogenous HDAC inhibitors that can directly induce Foxp3 expression and promote Treg cell maturation. This also elucidates the biological rationale behind the Carrimycin group's convergence toward the normal group in the PCoA plot. While traditional ecological paradigms suggest that high diversity equates to health, in this study, the reduced diversity indices in the Carrimycin group do not signify a deterioration of the microbiota; rather, they reflect the dominant expansion of specific beneficial taxa (*Akkermansia* and *Alistipes*) under high selection pressure. The improved microbial environment further downregulated the body's immune stress levels, decreasing Gr-1<sup>+</sup> cell infiltration and pro-inflammatory factor secretion. This combined regulation of the "Microbiota-Immune" axis establishes the pharmacological foundation for Carrimycin in the treatment of ulcerative colitis.

Conclusion

574 In summary, the present study demonstrates that carrimycin possesses remarkable  
 575 therapeutic efficacy against DSS-induced acute ulcerative colitis in mice. The protective  
 576 mechanisms are multi-pronged, involving the suppression of systemic pro-inflammatory  
 577 cytokines, the direct cell-autonomous correction of the mucosal Th17/Treg cell imbalance,  
 578 and the structural reconstruction of the gut microbiota via the eradication of pathogens  
 579 (Escherichia-Shigella) and the enrichment of key beneficial commensals (Akkermansia).  
 580 These insights suggest that carrimycin holds significant therapeutic promise as an  
 581 innovative, multi-target immunomodulatory candidate for the clinical management of  
 582 ulcerative colitis and related inflammatory bowel disorders.

583 Abbreviations

| Abbreviations | full name                                                         |
|---------------|-------------------------------------------------------------------|
| UC            | Ulcerative colitis                                                |
| IBD           | Inflammatory bowel disease                                        |
| CA            | Carrimycin                                                        |
| DSS           | Dextran sulfate sodium                                            |
| DAI           | Disease Activity Index                                            |
| H&E           | Hematoxylin and eosin                                             |
| Th17          | T helper 17                                                       |
| Treg          | Regulatory T cell                                                 |
| Foxp3         | Forkhead box P3                                                   |
| RORyt         | Retinoic acid receptor-related orphan<br>nuclear receptor gamma t |
| SCFAs         | Short-chain fatty acids                                           |
| LPS           | Lipopolysaccharide                                                |
| IL            | Interleukin                                                       |
| TNF-α         | Tumor necrosis factor-alpha                                       |
| IFN-γ         | Interferon-gamma                                                  |

|      |                                |
|------|--------------------------------|
| OTU  | Operational Taxonomic Unit;    |
| PCoA | Principal Coordinate Analysis. |

584

585 Ethics approval and consent to participate

586 All animal procedures were performed in accordance with the institutional guidelines  
587 and approved by the Institute of Life Science, eBond Pharmaceutical Technology Ltd.  
588 (Ethics Approval No.: eB-D2024015). The study did not involve human participants.

589 Consent for publication

590 Not applicable. This manuscript does not contain any individual person's data in any form.

591 Availability of data and materials

592 The 16S rRNA sequencing data have been deposited in NCBI SRA under BioProject  
593 accession PRJNA1480859 and are publicly available. he BioProject can be accessed  
594 directly via the following link:<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1480859>

595 Competing Interests

596 All other authors declare that they have no competing financial or non-financial interests.

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605 Authors' contributions

606 All authors contributed to the study conception and design. Material preparation and data  
607 collection were performed by Xia Tong, Hong Liu, Jie Lei, Pan congand Li zheng.Zi Wang and  
608 Xin Zhang supervised the experimental work and data analyses.The first draft of the

manuscript was written by Wanqin Wang and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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