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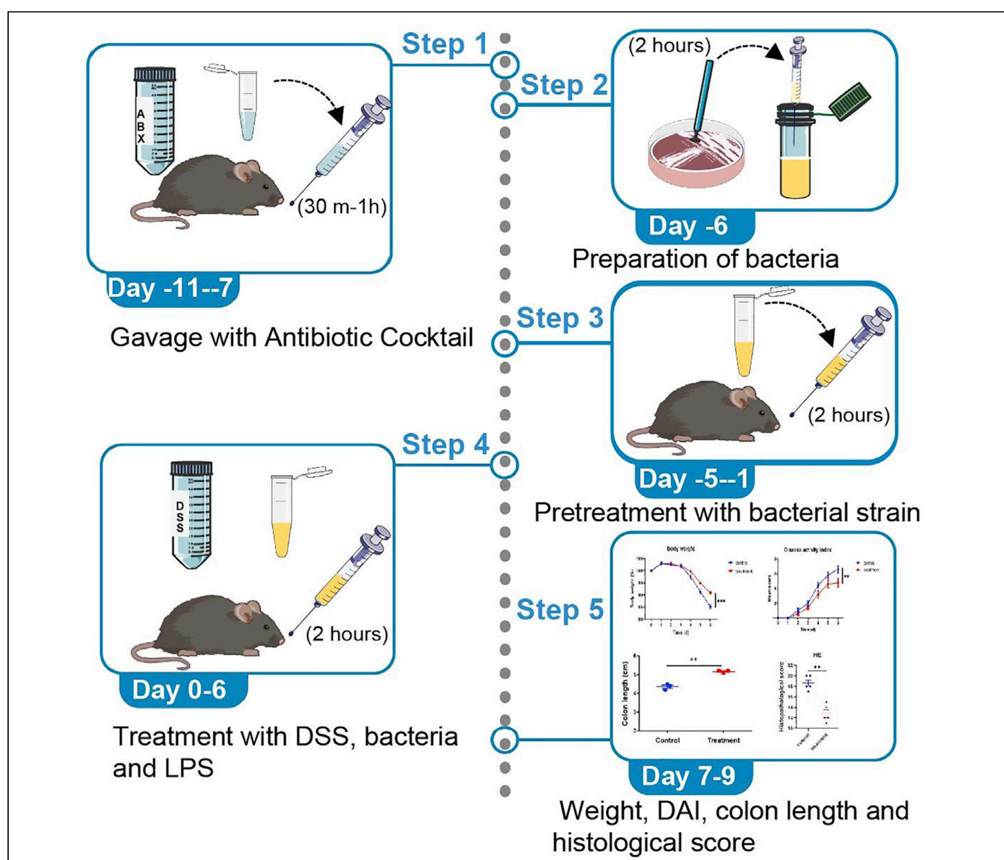
A mouse model to test the anti-inflammatory effect of facultative anaerobes on dextran sulfate sodium-induced colitis



The role of facultative anaerobic bacteria in colitis remains to be elucidated. We have adapted a mouse model to explore the effect of individual facultative anaerobic bacteria on colitis, focusing on adapting a cocktail of antibiotics and multiple instillations by gavage. Weight, disease activity index, colon length, and histological score are used to assess the severity of colitis. We also describe anaerobic processing protocols of preparing facultative anaerobes.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Kaiyuan Yu, Qiang Tang, Zhi Yao, Quan Wang

yaozhi@tmu.edu.cn (Z.Y.)
wangquan@tmu.edu.cn (Q.W.)

Highlights

Mouse model to explore the effect of facultative anaerobic bacteria on colitis

Optimized protocol for preparation and transplantation of facultative anaerobic bacteria

Analysis of bacterial effects on experimental colitis remission

Yu et al., STAR Protocols 4, 101988
March 17, 2023 © 2022 The Authors.
https://doi.org/10.1016/j.xpro.2022.101988

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A mouse model to test the anti-inflammatory effect of facultative anaerobes on dextran sulfate sodium-induced colitis

Kaiyuan Yu,[1,2,3] Qiang Tang,[1,2] Zhi Yao,[1,*] and Quan Wang[1,4,*]

[1]Key Laboratory of Immune Microenvironment and Disease (Ministry of Education), Tianjin Institute of Immunology, Tianjin Institute of Urology, Department of Immunology, School of Basic Medical Sciences, The Province and Ministry Co-sponsored Collaborative Innovation Center for Medical Epigenetics, Tianjin Medical University, Tianjin 300070, China

[2]These authors contributed equally

[3]Technical contact: yukaiyuan0419@163.com

[4]Lead contact

*Correspondence: yaozhi@tmu.edu.cn (Z.Y.), wangquan@tmu.edu.cn (Q.W.)
https://doi.org/10.1016/j.xpro.2022.101988

SUMMARY

The role of facultative anaerobic bacteria in colitis remains to be elucidated. We have adapted a mouse model to explore the effect of individual facultative anaerobic bacteria on colitis, focusing on adapting a cocktail of antibiotics and multiple instillations by gavage. Weight, disease activity index, colon length, and histological score are used to assess the severity of colitis. We also describe anaerobic processing protocols of preparing facultative anaerobes.
For complete details on the use and execution of this protocol, please refer to Li et al. (2022).[1]

BEFORE YOU BEGIN

Reagent preparation

Timing: 2 h preparation time, 4 h wait time

1. Prepare sterile phosphate-buffered saline (PBS) solution (pH 7.4).

   Note: PBS solution can be stored at 20°C–24°C for 1 week.

2. Add 0.1% L-cysteine and resazurin in the Brain Heart Infusion agar (BHI).
3. Add 10 mL of BHI to Schering bottles, and autoclave.

   Note: Prepare 10 mL Schering bottles (Figure 1).

   Note: Redox indicator resazurin, which turns to a clear color under anoxic conditions.[2] The color of BHI changed to purple when resazurin was added. After autoclaving, the BHI changes from purple to the original color.

   Note: BHI media can be stored under anaerobic conditions at 20°C–24°C for 2 months.

4. Add 100 mg lipopolysaccharides (LPS) to 50 mL sterile water, then divide into sterile 1.5-mL microcentrifuge tubes.

   Note: LPS solution can be stored under −20°C for 6 months.

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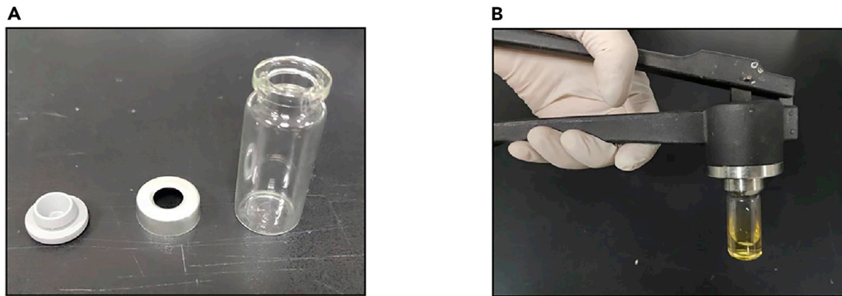

Figure 1. Prepare Schering bottles

(A) Prepare vials, butyl stoppers, and aluminum hollow sealing caps.

(B) Fill 10 mL BHI medium into vials and plug the bottle with a butyl stopper and cover with an aluminum hollow sealing cap. Use the sealing pliers to compress the aluminum hollow sealing cap to seal the vials.

The preparation of the standard growth curve for measuring CFU

Timing: 2 days

5. Streak facultative anaerobe on a BHI plate, and culture it in an anaerobic culture bag in the incubator for 24 h at 37°C.
6. Prepare bacteria for gavage (Figure 2).
   a. Pick a single colony from the BHI plate with tips.

   Note: The plate with colonies is stored at 4°C after use.

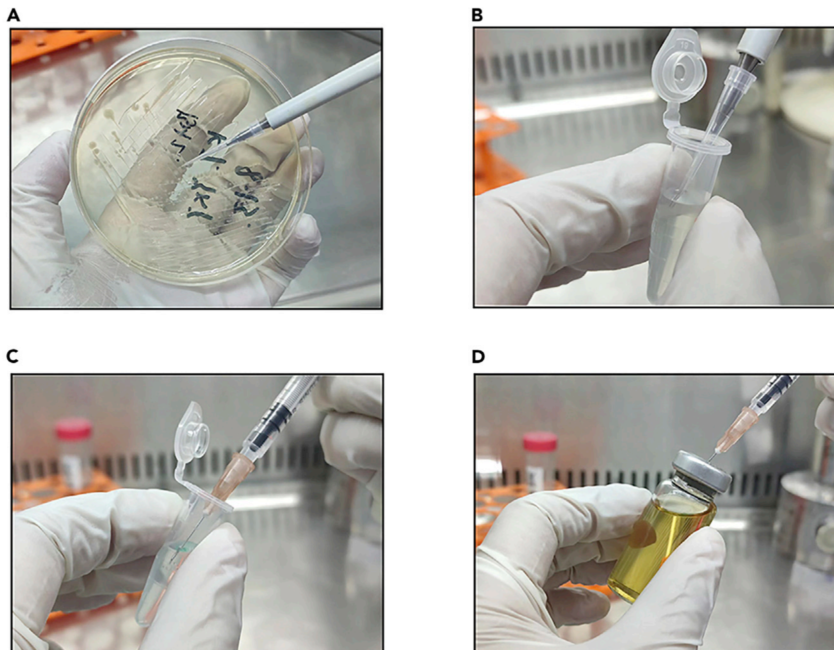

Figure 2. Bacterial inoculation process

(A) Pick a single colony from the bacterial culture dish.

(B) Suspend it in 1 mL sterile PBS.

(C and D) Using a syringe to inoculate 100 μL PBS suspended with bacteria into a vial containing BHI medium.

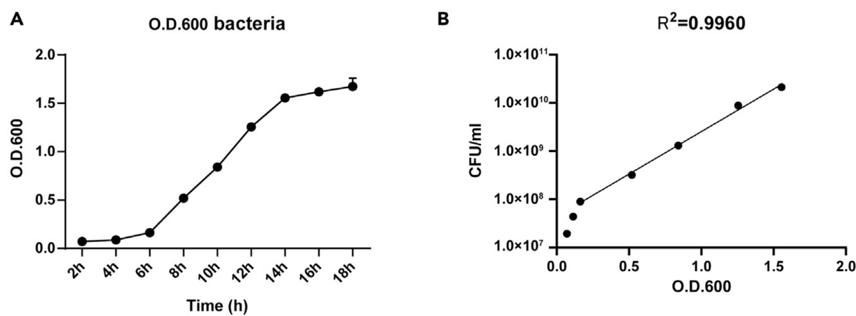

Figure 3. The standard curve for measuring bacteria CFU
(A) The example standard growth curve of bacteria.
(B) Correlation between the CFU and OD600. Data are represented as mean ± SEM.

Note: To ensure the bacteria's viability, the plate used to culture bacteria should be changed every week.

b. Suspend the colony in sterile PBS.
c. Inoculate it in 10 mL BHI broth, and shake the broth to mix well.
d. Statically culture at 37°C in a bacteriological incubator.

Note: The static culture is designed to mimic the growth environment of intestinal microorganisms.

7. Measure the concentration of bacteria by spectrophotometer at 600 nm of optical density (OD600).
   a. From the 2 h culture, take 1 mL and detect OD600, by the spectrophotometer every 2 h.
   b. Between the 10th and 16th hour, dilute bacteria solution in sterile PBS at a 1:10 ratio from 10^{-1} to 10^{-6} to make 1 mL in 1.5 mL sterile microcentrifuge tubes, one by one.
   c. Add 100 μL onto BHI plates in triplicate respectively, and incubate the plates in an anaerobic culture bag at 37°C overnight.
8. Count the CFU of the countable dots on the plate and determine the mean cell count from the triplicate experiments.

Note: Fewer than 500 colonies of one dilution is considered countable on the plate.

9. Draw the standard growth curve of the bacterial strain.
   a. Calculate the concentration of bacteria at each time point according to the dilution ratio.
   b. Statistically analyze the correlation of CFU and OD600, used for subsequent CFU calculation (Figure 3).

Bacterial inoculum preparation

Timing: 1 h preparation time, 24 h wait time

10. Pick a single colony and inoculate it in 10 mL BHI broth, statically culture for 14 h at 37°C in a bacteriological incubator.

Note: In order to ensure the best vitality of the bacteria, the bacteria should be in the logarithmic growth period when collecting bacteria. The logarithmic growth phase was determined according to the growth curve.

11. Calculate the CFU of bacteria.
    a. Harvest the facultative anaerobe at 8,000 g at 4°C for 5 min.
    b. Discard the supernatant and resuspend the pellet with sterile PBS buffer containing 0.1% L-cysteine at a proper concentration.

For example, if the OD600 of the bacterial strain culture medium is 0.7, the concentration will be 1×10^9 CFU/mL. If 10 mice are required to be given a gavage at the dose of 1×10^9 CFU/200 μL PBS per mouse, harvest 1 bottle (10 mL) of the culture medium, then resuspend the bacteria with 2 mL PBS containing 0.1% L-cysteine.

⚠ CRITICAL: 1. L-cysteine is used to keep the solution in a hypoxic state. 2. In order to ensure the effect of gavage, make sure that the total volume of PBS to resuspend the bacteria will not exceed 250 μL per mouse.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Agar	Solarbio	Cat# A8190
Brain heart infusion broth	Sigma	Cat# 53286
L-Cysteine	Sigma	Cat# 52-90-4
Phosphate buffered saline	Sigma	Cat# P3813
Metronidazole	Sangon Biotech	Cat# A600633-0025
Neomycin	Sangon Biotech	Cat# A610366-0025
Vancomycin	Sangon Biotech	Cat# A600983-0001
Ampicillin	Sangon Biotech	Cat# A610028-0025
Lipopolysaccharides	Sigma	Cat# L2880
Ethanol	Fisher Scientific	BP2818100
Xylene	Fisher Scientific	X3S-4
Experimental models: Organisms/strains		
6–8 weeks old SPF male mice	Charles River	C57BL/6
Software and algorithms		
GraphPad Prism 7.0	GraphPad	https://www.graphpad.com/
Other		
Petri dish	NEST	752001
10 mL Penicillin bottle	N/A	N/A
Butyl plug	N/A	N/A
Aluminum cap	N/A	N/A
1.5 mL EP Tube	Axygen	MCT-150-C
Gavage needle	Solomen	B0222-1
1 mL disposable sterilized syringe	N/A	N/A
Surgical scissors	N/A	N/A
Tissue forceps	N/A	N/A
Incubator	Yamato	IC412C
50 mL centrifuge tube	Bio Faith	Cat# 2400011
Spectrophotometer	Bio-Rad	samrtspec plus
Paraffin wax	Sigma-Aldrich	17310
Embedding cassette	CITOTEST	80106-1100-16
Rotary microtome	Leica	HistoCore BIOCUT R
Hematoxylin-eosin (HE) stain kit	Solarbio	G1120
Glass slides	Micro Slides	48311-703
4% paraformaldehyde	Solarbio	P1110
Resinous mounting medium	Solarbio	G8590

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Stool DNA Kit	Omega Bio-tek	D4015-02
Fume hood	N/A	N/A

⚠ CRITICAL: Exposure to xylene can irritate the eyes, nose, skin, and throat. Xylene can also cause headaches, dizziness, confusion, loss of muscle coordination. Pure ethanol can irritate the skin and eyes. Xylene and ethanol are volatile. Consequently, they must be operated in a fume hood.

Alternatives: For tissue fixation, 4% paraformaldehyde can be replaced by formalin.

MATERIALS AND EQUIPMENT

Antibiotic cocktail		
Reagent	Final concentration	Amount
Ampicillin	1 g/L	400 mg
Vancomycin	0.5 g/L	200 mg
Neomycin	1 g/L	400 mg
Metronidazole	1 g/L	400 mg
Milli-Q water	N/A	400 mL
Total	N/A	To 400 mL

Mice

All mice were on a C57BL/6 background, purchased from the Academy of Military Medical Science (Beijing, China). The mice were raised and maintained under specific pathogen-free conditions at Tianjin Medical University. All studies were approved by Animal Care and Use Committee at Tianjin Medical University.

STEP-BY-STEP METHOD DETAILS

The Schematic diagram of the animal model is shown in Figure 4.

Preparation of male mice for gavage

⏲ Timing: 6 days

⚠ CRITICAL: 1. 6–8 weeks old specific-pathogen-free (SPF) male mice must be kept in cages for three days to adapt to the new environment. 2. Mice need to be pre-treated with an oral mixture of antibiotics for five days before bacterial gavage. 3. Gavage procedures for antibiotics and bacteria cultures are identical (Figure 5).

This section describes the process of clearing bacteria from the gut and how to measure the effect of clearing bacteria.

1. From day -11 to day -7.
   a. On the first day of the model, in the animal facility, weigh male mice to be included in the model and randomly assign the animals into two groups: the control and treatment group.

⚠ CRITICAL: The two groups of mice are ensured to have similar activity and body weight.

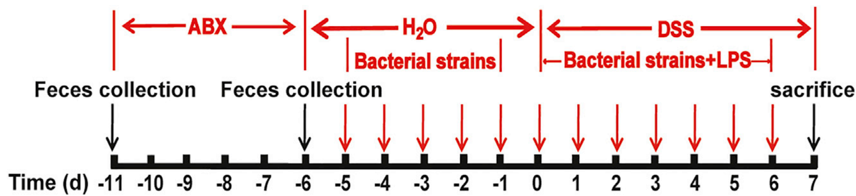

Figure 4. The schematic diagram for the mouse model
First, the mice were pretreated with the antibiotic cocktail for 5 days to eliminate the autochthonous microbiota and then given daily bacterial strains gavage for 5 days. The mice were treated with DSS for 7 days on day 0. During this period, the mice were given daily bacterial strains gavage and LPS challenge.

b. Collect at least 150 mg of feces into 1.5 mL sterile microtubes to evaluate the effect of antibiotic treatment and store them in a −80°C refrigerator.

Note: These stool samples can be processed with samples collected later.

⚠ CRITICAL: 1. When collecting samples, drain mouse urine to avoid contamination of feces by urine. 2. Place the microtubes with the collected feces on ice in time.

c. Add ampicillin (1 g/L), vancomycin (500 mg/L), neomycin (1 g/L) and metronidazole (1 g/L) (ABX) to sterile drinking water.

Note: ABX is used to clear most the intestinal bacteria (Kirkland et al.[3]).

Note: Metronidazole is difficult to dissolve in sterile water and should be shaken vigorously for about 5 min to help dissolve.

Note: The ABX is stable for two weeks at 20°C–24°C.

d. Orally gavage the mice with ABX.

Note: Normally, mice drink very little ABX, so they need to be gavaged to ensure adequate ABX into the intestine.

i. Grab and fix the mouse to straighten its head and neck.
ii. Insert the gavage needle (8#) parallel to the lower jaw until the throat (Figure 5A).
iii. Verticalize the needle and insert it along the esophagus without any obstruction, until almost the whole needle is inserted (Figure 5B).

Note: If there a is blockage, adjust the needle direction until smooth insertion.

e. Inject 500 μL of the ABX solution slowly into the esophagus without liquid overflowing from the mouth and causing choking.

Note: Gavage needles need to be immersed in 75% ethanol for sterilization after daily use.

2. On day -6.
   a. Collect at least 150 mg of feces into 1.5 mL sterile microtubes.
   b. Replace the ABX solution with sterile water.

Note: This step aims to reduce the concentration of ABX in the intestinal so that facultative anaerobe bacteria can colonize.

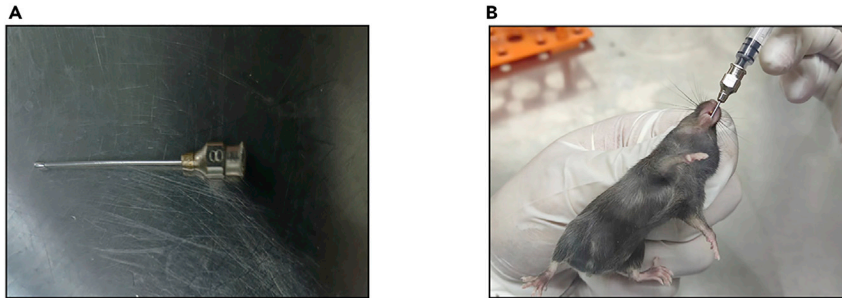

Figure 5. Oral gavage with antibiotics and bacteria
(A) The gavage needle.
(B) The diagram of oral gavage. Hold the skin on back, head and neck of the mouse to stabilize the body and keep the angle between the lower jaw and neck of the mouse at 180°to ensure that the esophagus is unobstructed. First, inject the needle from the corner of the mouse's mouth. When it passes through the throat, the resistance disappears obviously. Keep the gavage needle moving in parallel with the esophagus, and gently adjust the direction to pass in case of obstruction.

c. Use the Stool DNA Kit to extract microbial genomic DNA from two batches of feces samples according to the manufacturer's protocols, and then determine the amount of DNA.
d. Calculate the fecal DNA concentration of mice treated with ABX for five days or without (Figure 6).

Pretreated with a single bacterial strain

Timing: 5 days

This section describes the process of pretreatment with a single strain.

3. From day -5 to day -1.
   a. Orally gavage the mice with 1 × 10^9 CFU of bacteria in 200 μL PBS. Gavage procedure for bacteria is identical to that described in step 1.

   Note: Mice in the control group are gavaged with 200 μL PBS containing 0.1% L-cysteine.

   b. Inject 200 mL of bacteria Slowly into the esophagus without liquid overflowed from the mouth and choking.

Induction of colitis by DSS (dextran sulfate sodium) and LPS

Timing: 7 days

This section describes how to treat mice with a single strain during colitis caused by DSS and LPS.

4. On day 0.
   a. Record the weight of mice.
   b. Add 3% DSS to the drinking water of the mice to cause a mouse experimental model of acute colitis.

   Note: 3% DSS solution can be stored at 20°C–24°C for 3–4 days.

   c. Orally gavage the mice with 1 × 10^9 CFU of bacterial strain and LPS (8 mg/kg body weight).

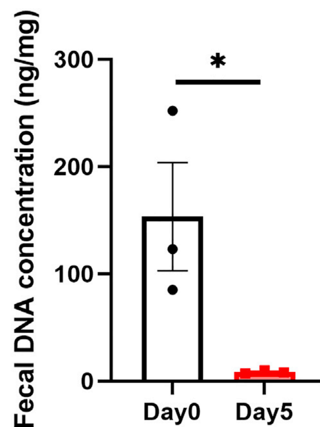

Figure 6. Fecal DNA concentration of mice before and after treatment with ABX for five days
Data are represented as mean ± SEM, Mann-Whitney test.

Note: Administration of DSS solutions to mice induced colitis which resembled mucosal pathology of human ulcerative colitis.[4] Colitis induced experimentally by DSS is a common model system to study etiology of inflammatory bowel disease. High concentrations of DSS can induce acute colitis.

5. From day 1 to day 6 troubleshooting 1, 2, 3.
   a. Record the weight of mice every day, analyze the body weight change according to the weight at day 0. Body weight loss was scored as follows: 0 (<2%), 1 (2%–5%), 2(5%–10%), 3 (10%–15%), 4 (>15%).[5]
   b. Observe the stool consistency and scored as follows: 0, normal; 1, mild soft stools; 2, very soft stools; 3, watery stools. Stool bleeding was scored as follows: 0, normal; 1, brown color; 2, reddish color; 3, bloody stool.
   c. Calculate disease activity index (DAI).

Note: DAI is the sum of stool consistence score, stool bleeding score and body weight loss score. DAI is a measure of the severity of colitis.

   d. Orally gavage the mice with bacteria and LPS every day.
   e. Supplement 3% DSS solution promptly to ensure that mice get it from day 1 to day 6.

Note: Broad-spectrum antibiotics (ABX) relieve colitis by reducing bacterial burden.[6] After ABX pretreatment, DSS-induced colitis was mild in this model. So LPS is used to aggravate colitis in the absence of commensal bacteria.[7]

Mouse dissection and harvesting colons

Timing: 1 day

This section describes how to harvest colons and perform tissue fixation.

6. On day 7.
   a. Record the weight and the stool score and calculate DAI (Figure 7).
   b. Perform cervical dislocation to euthanize and prepare for dissection.
   c. Soak the surface of the body in 70% vol/vol ethanol and wipe it off with paper.
   d. Use dissection scissors to open the abdominal cavity and remove the colon intact (Figure 8).

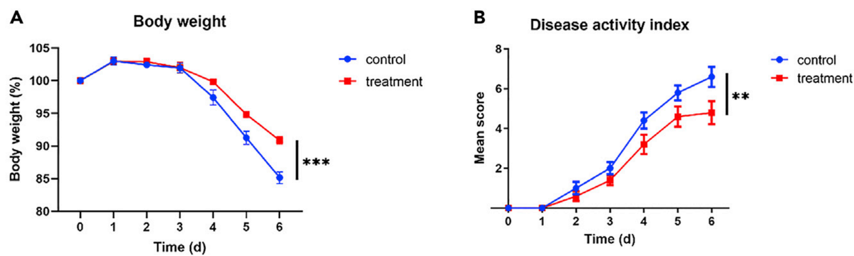

Figure 7. Mice treated with DSS, LPS, and single bacterial strain

(A) The body weight change during mice treated with DSS plus daily single bacterial strain gavage and LPS challenge.
(B) The disease activity index change during mice treated with DSS plus daily single bacterial strain gavage and LPS challenge. Data are represented as mean ± SEM, two-way ANOVA.

Note: The intact colon includes the cecum to the distal colon.

e. Photographed all colons against a blue background and measured for length (Figure 9).
f. Using a scissor, cut no less than 4 mm segments from a distal part of colon and then the segments of the distal part of colon is flushed with PBS using a gavage needle, doused in 4% paraformaldehyde at 20°C–24°C for fixation.

II Pause point: Colon tissue should be fixed in 4% paraformaldehyde for 1–2 days.

⚠ CRITICAL: 1. After around day 5–7 oral DSS, the feces may be difficult to observe because of the severe colitis. 2. Replenish DSS solution in time. 3. Dissection should be gentle and precise so as not to damage the colon. 4. Take a picture as quickly as possible to keep the tissue fresh.

Preparation of colons for cross-sectional hematoxylin-eosin staining

Timing: 1 day

This section describes the dewaxing, embedding, and sectioning of intestinal tissue.

7. On day 8 troubleshooting 4.
   a. Preheat a dry oven to 70°C.
   b. After 24 h at 20°C–24°C, discard the PFA and transfer the tissue to the embedding box. Mark with a pencil.
   c. Dehydrate specimens in graded ethanol solutions at 20°C–24°C.
      i. Place rack of specimens in 70% ethanol for 60 min.
      ii. Transfer rack of specimens to 85% ethanol for 40 min.
      iii. Transfer rack of specimens to 95% ethanol for 60 min.
      iv. Transfer rack of specimens to fresh 95% ethanol for 40 min.
      v. Incubate rack of specimens for 60 min in 100% ethanol.
      vi. Transfer rack of specimens to fresh 100% ethanol for 40 min.
   d. Place rack of specimens in xylene for 50 min and then flesh xylene for 50 min.
   e. Completely immerse the colon tissue in liquid wax in a regulated oven at 60°C for 30 min and then fresh wax for 60 min.

Note: Fresh xylenes, graded ethanol solutions and liquid wax should be replaced every two months to ensure the effect of wax immersion.

Note: The liquid wax should remain clear. If there is a solidified wax, it should be timely dissolved by heating.

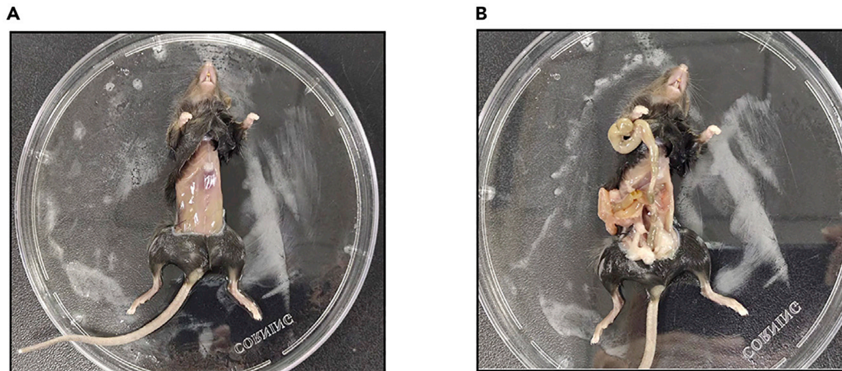

Figure 8. Sacrifice of mice
(A) Cut the abdominal skin of the mice to expose the peritoneum.
(B) Open the abdominal cavity and separate the small intestine and ileocecum at their junction. Isolate the ileocecum and colon and cut them at the anus.

f. Carefully embedded the intestinal specimens in paraffin. Cut tissue sections on a microtome at 5-μm thickness.

Pause point: The tissue in the wax block can be preserved for years.

Note: The hardness of the wax is selected based on the tissue type. The higher the tissue hardness, the higher the melting point of the wax should be. For general purposes (Such as embedding colon tissue), paraffin wax with a melting point of about 54°C–57°C is recommended.[8]

g. Use tweezers to pick up the sections and transfer to the 45°C water bath, and then take the floating sections using slides.
h. Melt paraffin to drive off any residual water trapped behind sections.
   i. Place slides in a xylene compatible, non-metal slide rack.
   ii. Bake the rack of slides in a 60°C oven for at least 1 h.

CRITICAL: 1. Markers whose writing is dissolved in organic solvents cannot be used for marking the embedding box. Pencil is recommended. 2. Deparaffinization and subsequent staining procedures should be carried out in the fume hood. 3. Make sure graded ethanol solutions and xylene completely submerges the tissue when dehydration.

Hematoxylin-eosin staining of intestinal tissue

Timing: 2–4 h

This section describes the process of hematoxylin-eosin staining, mounting and how to assess histopathological scores.

8. On day 9 troubleshooting 5.
   a. Dissolve surrounding and infiltrating paraffin from the sections. Perform all steps at 20°C–24°C.
      i. Place rack of slides in xylene for 10 min.
      ii. Transfer rack of slides to fresh xylene for 8 min.
   b. Clear the xylene from slides at 20°C–24°C.

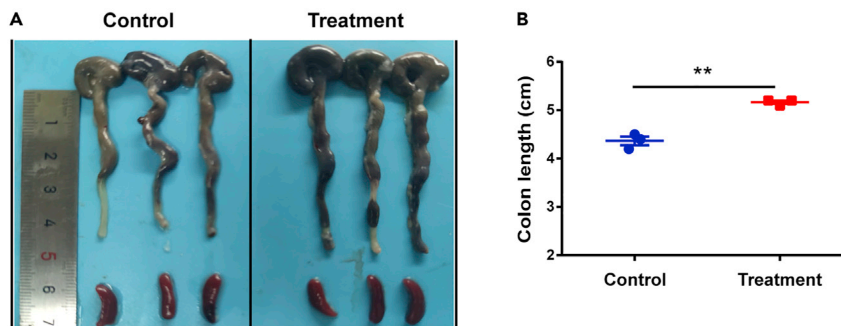

Figure 9. Comparison of colon length between treatment group and control group after DSS treatment
(A) The appearance of the colon and spleen.
(B) Colon length analysis. Data are represented as mean ± SEM, Mann-Whitney test.

   i. Place rack of slides in 100% ethanol for 2 min.
   ii. Transfer rack of slides to fresh 100% ethanol, and incubate for 1 min.
c. Dehydrate slides in three changes of ethanol at 20°C–24°C.
   i. Transfer slide rack to 95% ethanol and incubate 1 min.
   ii. Transfer slide rack to 85% ethanol and incubate 1 min.
   iii. Transfer slide rack to 70% ethanol and incubate 1 min.
   iv. Rinse slides in running tap water for 30 s.

Note: Xylene and ethanol used for staining can be reused. In order to take a clear image, we recommend replacing these solutions after processing every 100 slides.

d. Transfer the slides to a staining jar with hematoxylin to completely cover tissue section and incubate for 6 min to stain nuclei.
e. Rinse slides in running tap water to remove excess stain.
f. Transfer the slides to a staining jar with Eosin solution and incubate for 30 s.

Note: Replace fresh hematoxylin and Eosin solution after every batch of 100 slides.

g. Rinse the slides in running tap water till the water is clear.
h. Successively transfer the slides into staining jars.
   i. 70% ethanol for 2 s.
   ii. 85% ethanol for 4 s.
   iii. 95% ethanol for 20 s.
   iv. 100% ethanol, two changes of 1 min each.
   v. Xylene for 2 min twice.
i. Mount coverslips to the slides with a resinous mounting medium.
j. Observe (and photograph if necessary) H&E staining slides with an optical microscope to assess histopathological scores (Figure 10).

Note: The severity of inflammation was assessed in seven parameters: (A) extent of inflammation (score of 0–4), (B) extent of crypt damage (score of 0–4), (C) infiltration of neutrophils and lymphocytes (score of 0–3), (D) submucosal edema (score of 0–3), (E) loss of goblet cells (score of 0–3), (F) reactive epithelial hyperplasia (score of 0–3), (G) crypt abscesses (score of 0–2).[5,9]

EXPECTED OUTCOMES

In this mice model, to evaluate the effect of individual bacterial strain on experimental colitis, the mice microbiome is ablated, allowing for the establishment of added facultative anaerobe bacteria

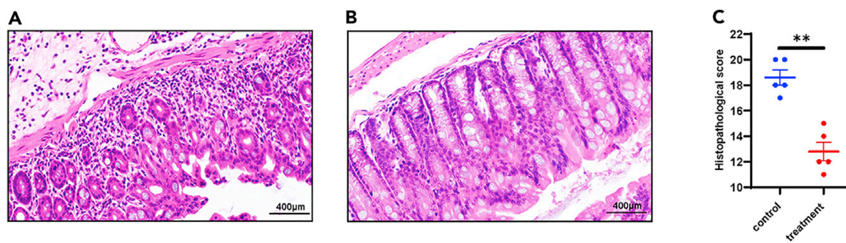

Figure 10. The severity of tissue inflammation assessment by H&E staining

(A and B) Representative images of H&E staining of colon tissue in the control group and treatment group.

(C) Histopathological scores are compared between the two groups. Data are represented as mean ± SEM, Mann-Whitney test.

in the intestine. Mice were pretreated with ABX for 5 days to ablate intestinal bacteria. Orally gavage the mice with 1 × 10^9 CFU of bacteria 1 day after ABX treatment for 5 days and mice were subsequently given the same strain in addition to 3% DSS and LPS challenge for 7 days (Figure 4).

Male mice gavaged with the useful facultative anaerobe bacterial strain show less weight loss, lower DAI score, longer colon length, and lower histopathological scores compared with the control group (Figures 7, 8, 9, and 10). The histological features of the colon from the two groups of mice are detailed in Figure 10B. Control group with severe colitis experience disruption of the colonic epithelial barrier and destruction of the intestinal villi.

LIMITATIONS

The model to test the effect of single bacterial strain on experimental colitis is dependent on the status of mice and microbiota. The diet, age, and gender of mice all influenced the results; thus the researchers should choose mice with the same background. Here we used the basic survival diet for mice.

ABX treatment is widely used to clear intestinal bacteria,[3] which helps to study the effects of individual bacteria. The method can be applied to test the effect of individual facultative anaerobe bacterial strain on DSS-induced colitis. However, the details of the model may vary for different strains.

TROUBLESHOOTING

Problem 1

Mice may die during treatment (step 5).

Potential solution

In this DSS-induced colitis model, wild-type mice would not die during the process. It is possible that improper oral gavage can cause mice to choke bacteria secondary to lung infection. As in Figure 5, the gavage feeding needle should insert into the esophagus without any obstruction.

Problem 2

The symptoms of colitis in mice are mild at day 5 or 6 (The DAI score is less than 5) (step 5).

Potential solution

Prolong the DSS processing time (no more than 9 days) or increase the concentration of DSS (we propose 3.5% instead).

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Problem 3

The feces are difficult to observe because of the dysentery (step 5).

Potential solution

Gently massage the abdomen of mice, then place them in a small container and observe.

Problem 4

The tissue is soft at the time of embedding (step 7).

Potential solution

The low concentration of alcohol results in inadequate tissue dehydration. Replace all alcohol promptly.

Problem 5

After H&E staining, the color of tissue is dull rather than a vivid red-blue (step 8).

Potential solution

Mount coverslips to the slides before the xylene volatilizes.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Quan Wang (wangquan@tmu.edu.cn).

Materials availability

This study did not generate unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS

This study was supported by grants from the National Natural Science Foundation of China (NSFC) programs (31970133, 32170186), the National Key Technologies R&D Program, Intergovernmental International Innovation Cooperation (2018YFE0102000), and the Science & Technology Development Fund of Tianjin Education Commission for Higher Education (2021KJ252).

AUTHOR CONTRIBUTIONS

All authors designed and analyzed experiments. K.Y. performed experiments. K.Y. and Q.T. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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