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TITLE:

Investigating Alterations in Caecum Microbiota After Traumatic Brain Injury in Mice

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KEYWORDS:

traumatic brain injury, caecum, microbiota, alterations, lateral fluid percussion injury, 16S-rDNA

SUMMARY:

Presented here is a protocol to induce diffuse traumatic brain injury using a lateral fluid percussion device followed by the collection of the caecum content for gut microbiome analysis.

ABSTRACT:

Increasing evidence shows that the microbiota-gut-brain axis plays an important role in the pathogenesis of brain diseases. Several studies also demonstrate that traumatic brain injuries cause changes to the gut microbiota. However, mechanisms underlying the bidirectional regulation of the brain-gut axis remain unknown. Currently, few models exist for studying the changes in gut microbiota after traumatic brain injury. Therefore, the presented study combines protocols for inducing traumatic brain injury using a lateral fluid percussion device and analysis of caecum samples following injury for investigating alterations in the gut microbiome. Alterations of the gut microbiota composition after traumatic brain injury are determined using 16S-rDNA sequencing. This protocol provides an effective method for studying the relationships between enteric microorganisms and traumatic brain injury.

INTRODUCTION:

Traumatic brain injury (TBI) is a global public health problem and the leading cause of death and disability in young adults[1,2]. TBI causes many deaths every year, and survivors experience a variety of physical, psychiatric, emotional, and cognitive disabilities. Therefore, TBI is a heavy burden to a patient's family and societal resources. TBI involves both the primary brain injury that occurs at the time of trauma and any secondary brain injuries that develop hours to months following initial injury. Secondary brain injury is mediated by several biochemical cascades, which are not only detrimental to the brain but also have significant negative effects on various organ systems, including the gastrointestinal system[3].

Currently, there are three models to induce TBI in animal experiments: fluid percussion injury, control cortical impact (CCI), and weight drop acceleration. Lateral fluid percussion injury (LFPI) is the most commonly used model to establish diffuse brain injury (DAI)[4]. The device produces brain injury through a craniectomy by applying a brief fluid pressure pulse to the intact dura. This pulse is created by the strike of the pendulum. LFPI is a reproducible and controllable modeling method for TBI research.

The microbiome is defined as the collective genomes of all microorganisms that reside in the human body. Intestinal microbes in particular not only play an important role in intestinal homeostasis and function but also regulate many aspects of host physiology and the functioning of other organs[5]. In recent years, there is increasing evidence that indicates that gut microbiota regulate brain development and function via brain-gut axes[6]. Disruption of the gut microbiota has been linked to several brain function disorders including Parkinson's disease, mood disorders, and autism[7]. Recently, preclinical studies have also reported that acute brain injury can induce changes in gut microbiota[8,9].

A study by Treangen et al.[10] found significant decreases in three microbial species and increases in two microbial species after CCI-induced TBI. This evidence indicates that modulation of gut microbiota may be a therapeutic method in TBI management. However, the mechanisms underlying brain injury-induced gut microbiota changes remain unknown. For this reason, a relatively simple and efficient model of studying the changes in gut microbiota after TBI is required. Therefore, the present study presents a protocol to examine alterations in gut microbiota after TBI in mice.

PROTOCOL:

All procedures performed were approved by the Experimental Animal Ethics Committee of Zhejiang University.

1. Animal care

1.1. Use 5- to 6-week-old male C57BL/6J mice (20–25 g of weight) in this experiment.

1.2. Maintain mice on a 12 h/12 h light/dark cycle, and make sure they receive food and water ad libitum. Provide the same amounts of food and water to both the sham and TBI groups throughout the study.

1.3. Make all efforts to minimize the animal pain and discomfort.

2. Induction of traumatic brain injury

2.1. Inject 5% chloral hydrate (6 mL/kg) intraperitoneally for anesthesia. Test the depth of anesthesia using an eye reflex or pain reflex. Use artificial tears or lubricant eye ointment to keep the eyes from drying.

2.2. After anesthesia, put the mouse in prone position. Use a temperature-controlled heating pad to maintain the temperature at 37 °C during the surgery and for 30 min after TBI.

2.3. Shave the hair of the incision area.

2.4. Disinfect the scalp with povidone iodine, then incise the scalp in a sagittal plane.

2.5. Use forceps to retract the incision on both sides and separate the periosteum slightly.

2.6. Use a marker to draw a circle (3 mm diameter) on the right parietal area of the skull, 2 mm away from the midline.

2.7. Drill the skull with an electric drill. Ensure that this step is operated carefully to protect the dura from being damaged.

2.8. Remove the bone flap and expose a small bone window (3 mm in diameter).

2.9. Place a plastic injury cannula (internal diameter = 2.5 mm, length = 8 mm) over the craniotomy and cement the cannula to the skull using a dental acrylic.

2.10. Fill the cannula with sterile 0.9% NaCl (normal saline) using a syringe (5 mL) to ensure that there are no bubbles in the cannula.

2.11. Turn on the oscilloscope and amplifier and ensure that the high-pressure tube of the lateral fluid percussion injury (LFPI) device is free of air bubbles. Test the device by delivering about 10 pulses until it gives a steady signal. Adjust the angle of the pendulum starting position to reach a pulse intensity of about 2.0 atm.

2.12. Connect the injury cannula to the LFPI device. Induce brain injury by pulling the trigger and releasing the pendulum. Then, obtain a pulse and transmit it to the dura through the

entire closed fluid-filled tubing system.

2.13. Operate the mice in the sham group with the same surgical procedure. Do not perform the LFPI.

3. Post-surgery treatment

3.1 After inducing the brain injury, remove the plastic cannula and suture the incision.

3.2 Lay the mouse on a heating pad until it is ambulatory. Set the temperature of the heating pad to 37 °C to accelerate anesthetic resuscitation.

3.3 Put the mouse back in the cage and administer food and water *ad libitum*.

4. Laparotomy and sample collection from the caecum

4.1 Sacrifice the mice by cervical dislocation at the corresponding time points.

NOTE: In this experiment, the chosen time points were 1 h, 6 h, 1 d, 3 d, and 7 d post-traumatic brain injury to analyze the dynamic evolution of gut microbiota.

4.2 Remove the hair from the surface of the abdomen. Disinfect the abdomen with iodine.

4.3 Place a sterile drape over the mouse. Make an incision from the lower abdomen midline, just above the prepuce in the male mice.

4.4 After the intestines are exposed, locate the cecum and gently separated it from other intestinal tracts. Avoid grasping the cecum with toothed or sharp forceps. Use atraumatic forceps, such as Adson forceps with serrations.

4.5 Cut the caecum with sharp scissors.

4.6 Extract the caecum contents manually onto sterile dressing and store the contents in 1.5 mL microcentrifuge tubes.

4.7 Store the caecum contents at -80 °C until microbiome analysis.

5. DNA extraction and 16S-rDNA sequencing and data analysis

5.1 Isolation of DNA from feces

NOTE: A commercially available DNA isolation kit (**Table of Materials**) was used for this

experiment.

5.1.1 Use a scalpel to scrape 300 mg of feces in a 2 mL microcentrifuge tube and place the tube on ice.

5.1.2 Add 1 mL of inhibit buffer to each sample. Vortex continuously for 1 min or until the feces sample is thoroughly homogenized.

5.1.3 Centrifuge the sample at the maximum speed for 1 min to pellet the feces particles.

5.1.4 Pipette 2 µL of proteinase K into a new 2 mL microcentrifuge tube. Pipet 600 µL of the supernatant from step 5.1.3 into the 2 mL microcentrifuge tube containing proteinase K. Then, add 600 µL of Buffer 1 and vortex for 15 s.

5.1.5 Incubate the sample at 70 °C for 10 min.

5.1.6 Add 600 µL of 100% ethanol to the lysate (1:1 ratio) and mix by vortexing. Centrifuge at the maximum speed briefly to remove drops from the inside of the tube lid.

5.1.7 Apply 600 µL of the lysate to the spin column. Centrifuge at the maximum speed for 1 min. Discard the flow-through. Repeat this step one more time. Then, transfer the column into a new 2 mL collection tube.

5.1.8 Open the spin column and add 500 µL of Buffer 2. Centrifuge at the maximum speed for 1 min. Remove the column and place it in a new 2 mL collection tube.

5.1.9 Add 500 µL of Buffer 3 into the column. Centrifuge at the maximum speed for 3 min. Discard the flow-through. Repeat the centrifugation process once to ensure the Buffer 3 is completely eluted.

5.1.10 Place the spin column into a new tube 2 mL collection tube and pipette 200 µL of Buffer 4 directly onto the membrane. Incubate for 1 min at room temperature (RT), then centrifuge at the maximum speed for 1 min to elute DNA.

5.2 16S-rDNA sequencing and data analysis

5.2.1 Use 20–30 ng of DNA to generate amplicons.

5.2.2 Use commercially available primers designed for the relatively conserved regions bordering the V3 and V4 hypervariable regions of bacteria 16S rDNA. The forward primers containing the sequence “CCTACGGRRBGCASCAGKVRVGAAT” and reverse primers containing the sequence “GGACTACNVGGGTWTCTAATCC” were used in the present study.

5.2.3 Make the PCR reactions mixture by adding 2.5 µL of Buffer 1, 2 µL of dNTPs, 1 µL of each primer, 0.5 µL of DNA polymerase, and 20 ng of template DNA in a tube. Use ddH_2O to adjust the reaction system to 25 µL.

5.2.4 Set the PCR reaction parameters as follows: perform the pre-denaturation at 94 °C for 3 min once. Perform denaturation at 94 °C for 5 s, anneal at 57 °C for 90 s, extend at 72°C for 10 s, and repeat this 24x.

5.2.5 Perform PE250/300 paired-end sequencing according to the manufacturer's instruction and use QIIME data analysis package for 16S rRNA data analysis.

NOTE: In this experiment, the DNA sequencing and data analysis were primarily done by a professional sequencing company.

REPRESENTATIVE RESULTS:

Establishment of TBI is shown in **Figure 1**. After anesthesia and disinfection, the scalp was incised sagittally (**Figure 1A**). A craniotomy (3 mm in diameter) was trephined into the skull over the right parietal cortex with an electric drill, the dura was kept intact (**Figure 1B,C**). A plastic injury cannula was placed over the bone window and cemented to the skull using dental acrylic (**Figure 1D**).

The procedure of lateral fluid percussion is shown in **Figure 2**. Before starting the device was tested by delivering about 10 pulses until it gives a steady signal. The angle of the pendulum was adjusted to the starting position to reach a pulse intensity of about 2.0 atm (**Figure 2A**). The cannula was filled with the sterile normal saline. Then the cannula was connected to the LFPI device (**Figure 2B**). Brain injury was induced by creating an impulse into the closed cranial cavity (**Figure 2C**).

Laparotomy and caecum fecal sample collection are shown in **Figure 3**. The laparotomy was performed on the lower abdomen and along the midline (**Figure 3A**). The caecum was identified and gently separated (**Figure 3B,C**). The caecum is usually located in the lower right part of the abdomen. It was then incised with sharp scissors (**Figure 3D**). The contents of caecum (**Figure 3E**) were extracted and stored in 1.5 mL tubes (**Figure 3F**). Fecal samples were immediately stored at -80 °C before further use.

16S-rDNA sequencing demonstrated reduced diversity of caecum microbiota in mice 3 days after TBI, the most abundant taxa in caecum contents of sham and TBI groups were showed in **Figure 4.** The Wilcoxon rank sum test was performed to evaluate microbiota differences between TBI and sham groups in the 16S sequencing analysis, and the *p* value was less than 0.05. The non-metric multi-dimensional scaling (NMDS) also showed changed composition of

caecum microbiota after TBI (**Figure 5**).

FIGURE LEGENDS:

Figure 1: Establishment of TBI. (**A**) After anesthesia and disinfection, the scalp was incised. (**B**) Operate a circinate craniotomy on the skull over the right parietal cortex. (**C**) Keep the dura intact. (**D**) Cement the plastic injury cannula to the skull using dental acrylic.

Figure 2: The procedure of lateral fluid percussion. (**A**) Adjustment of the pendulum starting position angle. (**B**) Filling of the cannula with sterile normal saline, then connection of the cannula to the LFPI device. (**C**) Brain injury induction by release of the pendulum and creation of an impulse into the closed cranial cavity.

Figure 3: Laparotomy and caecum fecal sample collection. (**A**) Beginning of the laparotomy from lower abdomen and along the midline. (**B,C**) Identification of the caecum and subsequent (gentle) removal. (**D**) Cutting of the caecum with sharp scissors. (**E**) Extraction of the contents of caecum. (**F**) Storing of the caecum content samples in 1.5 mL Eppendorf tubes.

Figure 4: The comparison of caecum microbiota diversity. Most abundant taxa in caecum content of sham and TBI groups demonstrated reduced diversity of caecum microbiota in mice 3 days after TBI.

Figure 5: The NMDS analysis. Non-metric multi-dimensional scaling (NMDS) showed changed composition of caecum microbiota after TBI.

DISCUSSION:

Presented here is a simple and efficient protocol to determine changes in cecal microbiota after TBI in mice. Induction of brain injury and collection of caecum content samples are critical parts of the protocol.

Despite researchers having studied the changes of gut microbiota following TBI, the brain injury used in these studies were CCI-[8] and weight drop/impact-induced models[9]. However, the CCI model mostly replicates brain contusion, and the weight drop model may suffer from some inaccuracies. Among all existing brain injury models, LFPI is the most commonly used model, which includes focal and diffuse brain injury[11,12] and replicates many important features observed in clinical TBI patients. Key steps throughout the process include protection of the dura and keeping the cannula airtight. However, both craniotomy and conglutination require the operation of a skilled performer, which may be a limitation of this protocol. However, LFPI is still a simple, stable, and accurate method. Therefore, TBI in mice was induced using LFPI in this study, which makes the protocol more representative and valuable in brain injury research.

For gut microbiota analysis, fecal samples were used for its non-invasiveness and convenience. Nevertheless, there are some limitations of fecal sampling. First, the amount of feces collected in each mouse at a certain time point is small, and the amount of feces may be insufficient to perform 16S-rDNA analysis. Second, due to post-traumatic gastrointestinal dysfunction, feces samples are difficult to collect during the acute phase after brain injury (1 h post-injury in this study). In addition, evidence has shown that fecal microbiota composition is different from microbiota in other intestinal segments[13]. Therefore, caecum contents were collected for gut microbiota analysis in this study. Caecum content sampling requires that mice are sacrificed, and this method allows for the sampling of intestinal tract for histological and immunological examinations, which are important research aspects in studies of brain injury-induced gut dysfunction. Additionally, this method can be used to investigate microbiota changes in other gastrointestinal tracts including the jejunum, ileum, and colon. In conclusion, this protocol is an ideal method for studying TBI-induced changes in gut microbiota.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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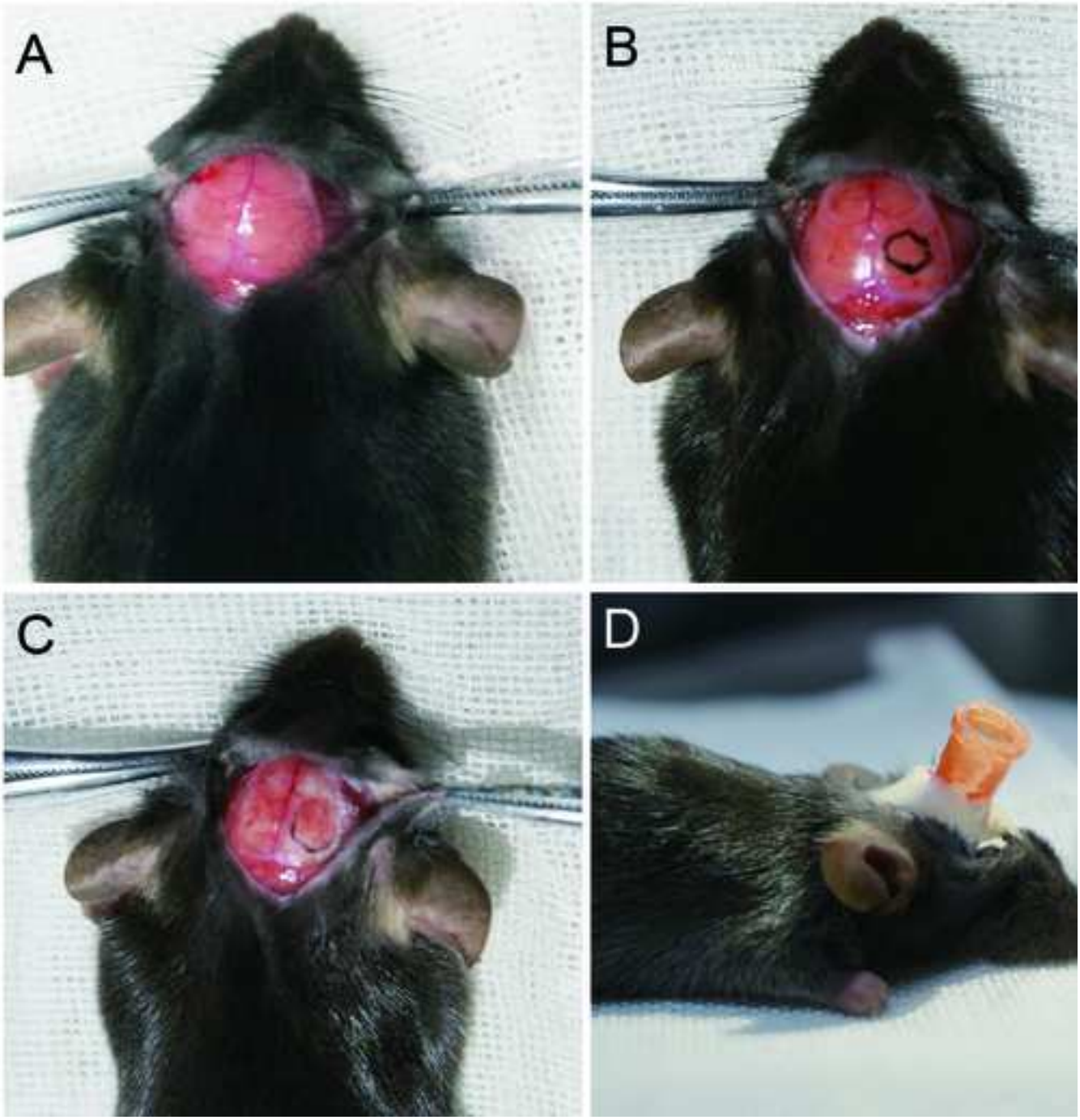

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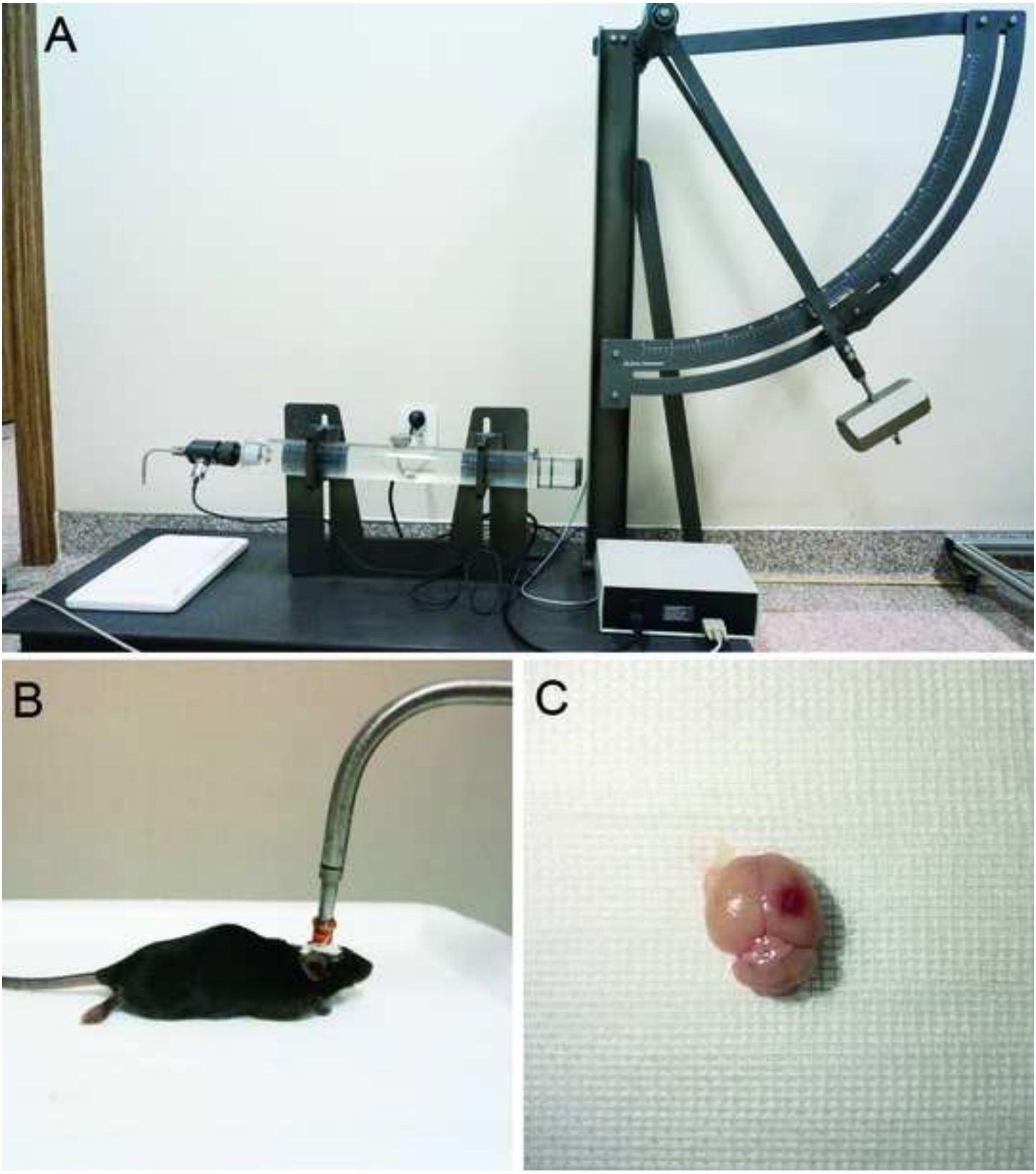

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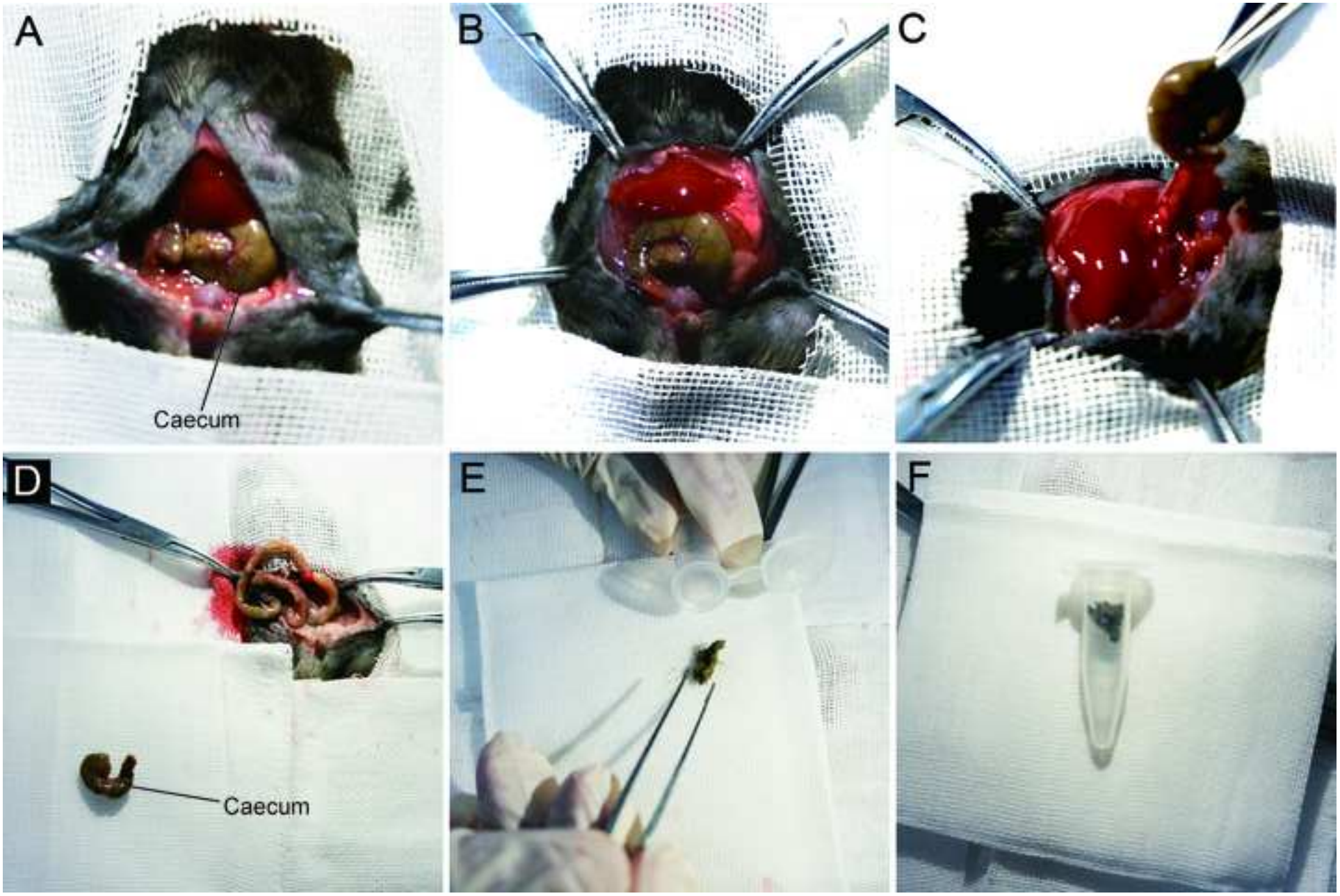

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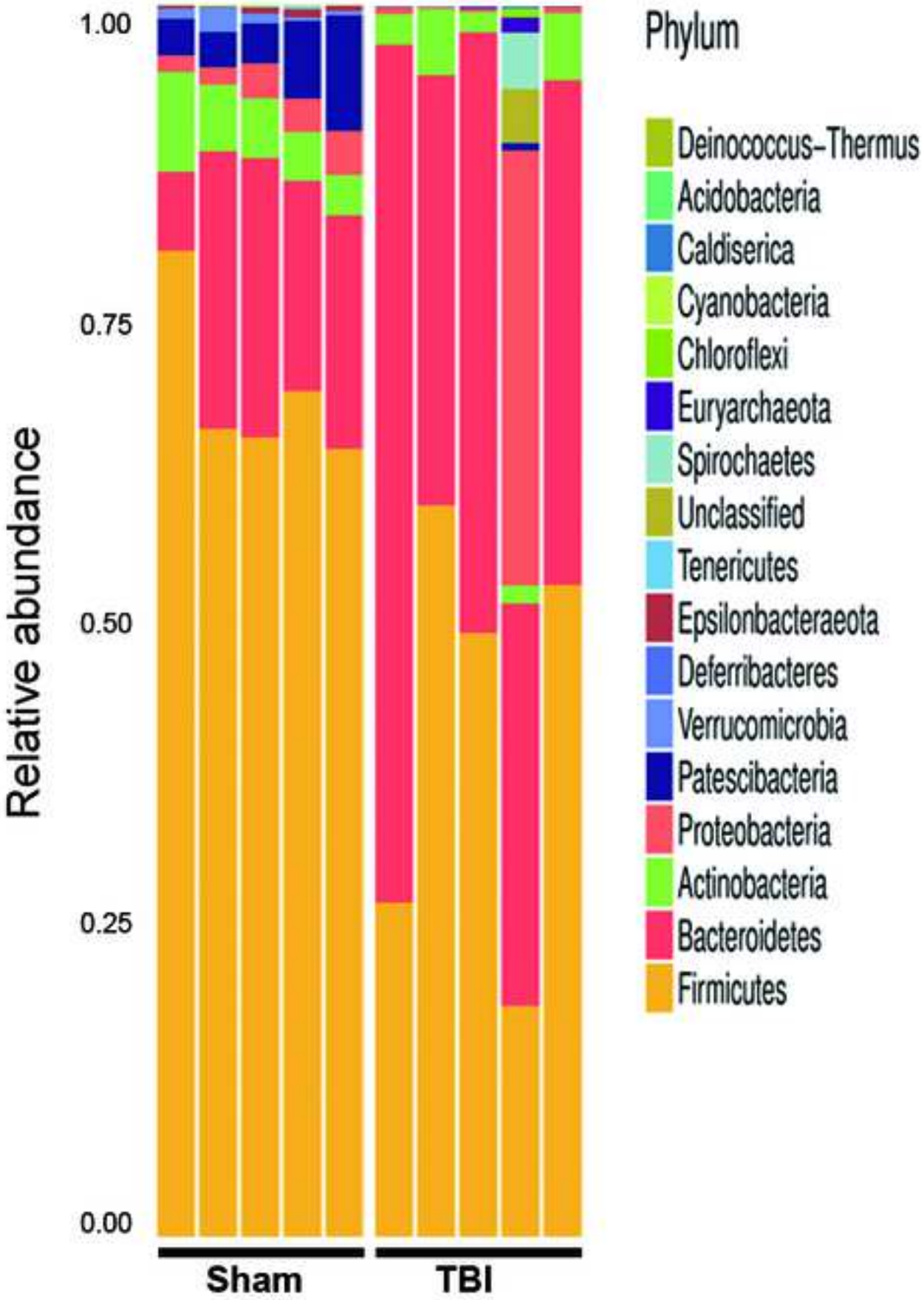

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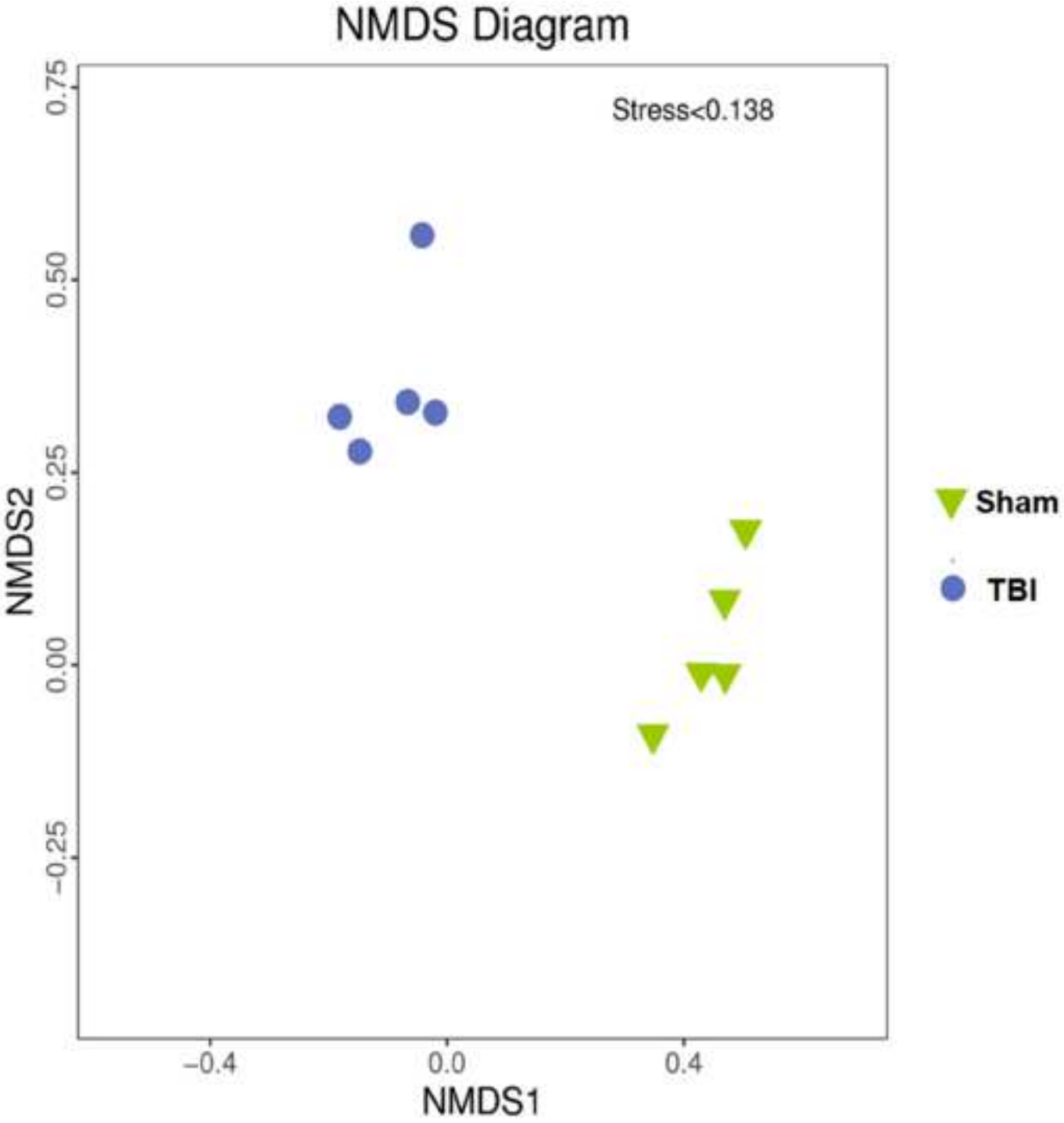

Table of materials

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Name of Material/ Equipment	Company
DNA isolation kit	QIAGEN
Gene analysis service	GENEWIZ
Heating pad	Shanghai SAFE Biotech Co.
Injector	The First Affiliated Hospital, School of Medicine, Zhejiang
LFPI device	Virginia Commonwealth University
Micro cranial drill	RWD Life Science
Povidone Iodine	The First Affiliated Hospital, School of Medicine, Zhejiang

Catalog Number	Comments/Description
51604	For fast purification of genomic DNA fror
	Gene analyse service
TR-200	heating pad
	injector
FP302	LFPI device
78061	Micro cranial drill
	Povidone Iodine

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Dear Editor,

Thanks for your email dated March 12, 2019 for our revised manuscript (Manuscript ID JoVE59410R1). We would like to express our gratitudes for your excellent work of rewording our manuscript and giving several valuable comments.

We have revised the manuscript according to comments marked in the manuscript.

We have also responded point by point to the comments as listed below. A revised version with the correction sections red marked was attached.

If you have any questions, please contact us without hesitation. We hope that the revised version is acceptable for publication, and we look forward to hearing from you soon.

Yours sincerely
Xiaofeng Yang

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Replies to Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

Reply：Thank you for such careful and meticulous revisions. We have seen the revised content, including the title and the short abstract, etc. It is indeed more concise than the previous version. Then, we have confirmed that the existing format was not changed during the entire revise process.

2. Please address specific comments marked in the manuscript.

Reply: All specific comments marked in the manuscript have been revised as required. Details were marked red in the manuscript.

3. Please expand the protocol section to show how the caecum content was used for sequencing.

Reply：Thank you very much for your instructive comments. We have expanded the protocol section. We described how to extract DNA from the caecum content and how the DNA amplification was done. Then we briefly described the 16S-rDNA sequencing and the data analysis.

4. Once done, please proofread the manuscript for any grammar or spelling issues.

Reply：Thank you for your suggestions. We reviewed the entire article again and corrected several grammar and spelling issues.