**ORIGINAL ARTICLE**

**Clostridium butyricum** exerts a neuroprotective effect in a mouse model of traumatic brain injury via the gut-brain axis

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**Abstract**

**Background:** Traumatic brain injury (TBI) is a common occurrence following gastrointestinal dysfunction. Recently, more and more attentions are being focused on gut microbiota in brain and behavior. Glucagon-like peptide-1 (GLP-1) is considered as a mediator that links the gut-brain axis. The aim of this study was to explore the neuroprotective effects of *Clostridium butyricum* (Cb) on brain damage in a mouse model of TBI.

**Methods:** Male C57BL/6 mice were subjected to a model of TBI-induced by weight-drop impact head injury and were treated intragastrically with Cb. The cognitive deficits, brain water content, neuronal death, and blood-brain barrier (BBB) permeability were evaluated. The expression of tight junction (TJ) proteins, Bcl-2, Bax, GLP-1 receptor (GLP-1R), and phosphorylation of Akt (p-Akt) in the brain were also measured. Moreover, the intestinal barrier permeability, the expression of TJ protein and GLP-1, and IL-6 level in the intestine were detected.

**Results:** Cb treatment significantly improved neurological dysfunction, brain edema, neurodegeneration, and BBB impairment. Meanwhile, Cb treatment also significantly increased the expression of TJ proteins (occludin and zonula occluden-1), p-Akt and Bcl-2, but decreased expression of Bax. Moreover, Cb treatment exhibited more prominent effects on decreasing the levels of plasma d-lactate and colonic IL-6, upregulating expression of Occludin, and protecting intestinal barrier integrity. Furthermore, Cb-treated mice showed increased the secretion of intestinal GLP-1 and upregulated expression of cerebral GLP-1R.

**Conclusions:** Our findings demonstrated the neuroprotective effect of Cb in TBI mice and the involved mechanisms were partially attributed to the elevating GLP-1 secretion through the gut-brain axis.

**KEYWORDS**

*Clostridium butyricum*, glucagon-like peptide-1, gut brain axis, neuroprotection, traumatic brain injury

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**1 | INTRODUCTION**

Traumatic brain injury (TBI) is acquired from an external force, which can inflict devastating effects to the brain vasculature and neighboring neuronal cells. Following mechanical and structural primary insults, a complex set of secondary injuries cascades exacerbated the brain injury and cognitive deficits. There are increasing evidences that cerebral edema and neural apoptosis after TBI could result in BBB impairment and the neurodegeneration. These conditions are related...
pathologically through multiple interacting mechanisms. Accounting for the fact that TBI is associated with these mechanisms, it is important to explore neuroprotective agents that may attenuate TBI.

Gastrointestinal dysfunction is one of several complications in patients with TBI. TBI can result in increased intestinal permeability and structural and functional damage of the gastrointestinal tract. There is evidence that intestinal bacteria are directly involved in the development of TBI. The gut microbiota is an important internal environment factor that regulates the bidirectional commutation that underlies the gut-brain axis. Indeed, microbiota dysfunction is linked to a variety of disorders such as experimental stroke, Parkinson’s disease, and Alzheimer’s disease. The gastrointestinal hormonal signaling pathway is one part of the complex network of communication between gut microbiota and the brain.

Glucagon-like peptide-1 (GLP-1) is released by intestinal L-cells, which predominantly localized in the colon and ileum. Gut microbiota changes were associated with variations of GLP-1 content. Gut microbiome are in direct contact with L-cells in the intestine. Thus, it is clear that different microbial populations may react to local environmental cues that can be regulated by the gut microbiota. While the composition of the gut microbiota varies greatly among individuals, alterations to the balance of common gut microbes may affect production of the short-chain fatty acids (SCFAs) butyrate, propionate, and acetate, which are products of intestinal bacterial fermentation. The levels of SCFAs in the intestinal tract are influenced by the microbial composition. SCFAs are thought to influence GLP-1 release via the activation of G-protein coupled cell surface receptors, G-protein coupled receptor (GPR) 41 and GPR43. GLP-1 receptor (GLP-1R) is found throughout the central nervous system (CNS), and GLP-1 signaling is implicated in the regulation of a variety of CNS functions, including neurotrophic and neuroprotective effects. Recent studies have shown that the injectable GLP-1 analog, liraglutide, significantly improved outcomes after severe brain trauma in mice, mitigating cognitive impairments after mild TBI in a mouse weight-drop model, which could cross the BBB to exert anti-inflammatory effects on cerebral endothelial cells and to prevent injury-induced changes in gene expression, including pathways associated with oxidative stress and neuroinflammation. GLP-1 is considered one of the chemical mediators that link the gut-brain axis. GLP-1 can engage multiple kinase signaling cascades including phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), protein kinase A (PKA), and extracellular signal-regulated kinase 2 (ERK2). Therefore, modulating the gut microbiome by promoting interactions through the use of probiotics could increase GLP-1 secretion and enhance its beneficial effects.

Clostridium butyricum (Cb) is a butyrate-producing probiotic and an effective agent known for its effects on intestinal bacteria-related diarrhea. In our previous studies, it was found that Cb is effective in the treatment of vascular dementia and diabetes combined with cerebral ischemia. Our previous studies showed that Cb can produce a large amount of SCFA butyrate in the gut and exert anti-apoptosis and antioxidant in vascular dementia animal models, which may have beneficial effects on host brain injury. These effects of Cb can mainly be explained by its ability to increase the production of butyrate and the secretion of the gut hormone GLP-1. Although the ability of Cb to attenuate neuroinflammatory responses and apoptosis and to promote GLP-1 have been separately reported in different experimental models, the effects of Cb on GLP-1 secretion and production have not been clarified in a TBI animal model.

In this study, we provide evidence supporting Cb as a treatment for and management strategy against TBI. We examined the hypothesis that Cb can exert neuroprotection against neurological dysfunction, brain edema, neurodegeneration, and BBB impairment in a mouse model of TBI, which were associated with an increase in the levels of GLP-1 via gut-brain axis.

### 2 | MATERIALS AND METHODS

#### 2.1 | Animal and bacteria preparation

Male C57BL/6 mice (18-22 g, 6-8 weeks old) were purchased from the Experiment Animal Centre of Wenzhou Medical University, Zhejiang, China. The animals were housed in groups of five mice per cage under constant temperature (23 ± 2°C), constant humidity (55 ± 5%), and a 12-hour light/dark cycle. Standard food and water were available ad libitum. Mice were allowed to adapt to the laboratory for 5-7 days before undergoing experimental procedures. All experiments were approved and performed in strict accordance with the guidelines of the Institutional Animal Care Committee of Wenzhou Medical University. C. butyricum WZMC1016 (CGMCC 9831) was provided by the China General Microbiological Culture Collection Center. The bacterial solution was prepared every day in sterile phosphate buffered saline (PBS) and administered orally at a concentration of 10⁸ CFU/day/mice as described previously.

#### 2.2 | Induction of TBI

The TBI mouse model was induced using a modified weight-drop impact head injury, as previously described and as in our previous study. Briefly, mice were anesthetized with 3.5% chloral hydrate via the peritoneum and fixed on an operating table. A midline longitudinal

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**Key Points**

- *Clostridium butyricum* significantly improved neurological dysfunction, brain edema, neurodegeneration, and BBB impairment.
- *Clostridium butyricum*-treated mice showed increased the secretion of intestinal GLP-1 and upregulated expression of cerebral GLP-1R.
- Our findings demonstrated the neuroprotective effect of *Clostridium butyricum* in TBI mice and the involved mechanisms were partially attributed to the elevating GLP-1 secretion through the gut-brain axis.
incision was made on the top of the skull and a bone window 3 mm in diameter was created to expose dura mater 2 mm rightward of the sagittal suture and 3 mm posterior to the coronal suture. A 20-g weight was dropped from a height of 20 cm onto the target area, after which the scalp wound was closed using standard suture material. Placebo animals underwent the same procedures but did not undergo the weight-drop impact head injury.

2.3 | Experimental design

After acclimation for 1 week, the animals were randomly divided into three groups (n = 25 for each group): (i) sham group (sham-operated control), (ii) TBI group (vehicle-treated TBI), and (iii) Cb group (Cb-treated TBI). Mice in the Cb group were intragastrically administered with Cb (10^9 CFU/ml) once daily for 14 consecutive days prior to the onset of TBI, and then following TBI, they were intragastrically treated once daily for 14 consecutive days. Mice in both the TBI and sham groups received an equal volume of saline. Mice in all three groups received a normal diet and were fed daily with a restricted diet (10% less than the daily ration) in order to reach the same intake degree. After the behavioral test, mice were sacrificed. Four to six mice from each group were deeply anesthetized and euthanized by cervical decapitation. The whole brain tissue was quickly removed and placed on ice. The injury cortex and a brain region adjacent to the injury were quickly dissected out and frozen in liquid nitrogen. In addition, serum was obtained by the centrifugation of blood samples and maintained at −80°C until analysis. Afterward, a colonic tissue sample from the proximal colon was collected and frozen immediately in liquid nitrogen. At the same time, tissue samples of the brain and the proximal colon (approximately 1.0 cm each) from three mice in each group were collected and fixed, paraffin-embedded, and sectioned at 5 μm. A diagram to describe these procedures is shown in Figure 1.

2.4 | Behavioral assessment

Post-trauma neurological deficits were evaluated in a blinded fashion using a 10-point NSS, as described previously. This assessment is composed of 10 individual tasks that are used to assess motor functions, balance, and alertness of the mice. One point is awarded for failing to fulfill an individual task and no point for succeeding. A higher score indicates a more severe neurological deficit. The test was performed at 1, 4, 24 hour, 4, 7, 10 and 14 days after trauma.

2.5 | Brain water content

Brain water content was measured according to a previous study with a minor modification. In brief, the mice were decapitated under deep anesthesia 24 hour after trauma. Brain samples were immediately harvested, and the injured hemispheres without the cerebellum or the olfactory bulb were accurately weighed (wet weight). Then, the brain samples were dried in a desiccating oven at 90°C for 48 hour and weighed again (dry weight). The percentage of brain water content (%) was calculated according to the wet-to-dry ratio ((wet weight – dry weight)/wet weight × 100).

2.6 | Fluoro-Jade C staining

Fluoro-Jade C staining is a reliable marker that exhibits a great affinity for degenerating neurons and has been widely applied in the study of brain trauma. The Fluoro-Jade C staining procedure was performed as described in our previous study. At 24 hour post-trauma, mice were euthanized with an overdose of 3.5% chloral hydrate and then transcardially perfused with 40 mL 4% paraformaldehyde (PFA). Brain samples were removed and postfixed for at least 1 day in the same

FIGURE 1 The experimental schematic diagram. (A) Mice were treated with C. butyricum for 28 days, including 14 days prior to the onset of TBI. (B) Neurological deficits, BBB impairment and intestinal barrier impairment were evaluated. C. butyricum, Clostridium butyricum; BBB, blood-brain barrier; GLP-1, Glucagon-like peptide-1; GLP-1R, GLP-1 receptor

- IL-6 level
- GLP-1 level
fixative solution. Brains were then paraffin embedded and cut at a thickness of 5 μm using a rotary microtome.

2.7 | BBB permeability assay

BBB permeability was assessed by measuring the intracerebral amount of Evans blue (EB) dye, as previously described.34 EB solution (2% in saline, 3 mL/kg body weight) was slowly injected into the tail vein at 24 hour following TBI and then circulated for 60 minutes. After being anesthetised, the mice were perfused through the left ventricle of the heart with cold saline to remove the intravascular dye. Next, the brains were removed, photographed and weighted. Then, the ipsilateral hemisphere was homogenized in formamide, soaked for 72 hour at 37°C, and centrifuged for 20 minutes at 12 000 g. The supernatant was collected and its optical density (OD) was recorded using an ultraviolet spectrophotometer at a wavelength of 625 nm. Calculations were based on external standard readings (80 μg/mL of EB dissolved in saline with eight serial dilutions). The amount of EB in the test sample was expressed in ng/g wet brain tissue. A higher amount of dye in brain tissue represents a greater vascular permeability and more severe BBB impairment.

2.8 | Immunohistochemistry and immunofluorescence

For immunofluorescence (IF), brain and colon samples were fixed with 4% PFA. Following deparaffinization, rehydration in a series of decreasing concentrations of alcohol, and antigen retrieval, the brain sections were blocked in PBS containing 5% normal bovine serum for 1 hour at room temperature and incubated with primary antibodies against occludin (Novus Biologicals, #NBP1-87402, Littleton, CO, USA) and ZO-1 (Santa Cruz Biotechnology, #sc-10804, Santa Cruz, CA, USA) at 4°C overnight. The sections were washed with PBS and incubated with the appropriate Alexa 488-conjugated species-specific secondary antibody (Chemicon, Rolling Meadows, Illinois, USA) for 2 hour at room temperature. The region around the injured cortical area was observed with an Olympus BX-51 fluorescence microscope (200× magnification; Olympus, Tokyo, Japan) and photographed using the Olympus soft image viewer.

For immunohistochemistry (IHC), Mice were sacrificed and the brain were removed, fixed in 4% formaldehyde and embedded into paraffin. The brain tissues were sliced at 5 μm. The progress of Nissl’s staining was performed according to our previous study.27 The colon was fixed with 4% PFA. GLP-1 primary antibody (1:250, Abcam, #ab33329, Cambridge, UK) was applied overnight at 4°C and incubated with HRP-conjugated secondary antibodies (1:2000, Beyotime Institute of Biotechnology, Shanghai, China), and the sections were visualized using diaminobenzidine (DAB) as the chromogen. Sections were observed with a light microscope (200× magnification; Nikon, Tokyo, Japan) and brown granules in cells observed under the microscope were defined as positive signals.

2.9 | Western blot analysis

At 14 days post-trauma, the mice were sacrificed and brain and colon samples were quickly removed. The ipsilateral cortex around the injured site and proximal colon were isolated and stored at −80°C until further use. The samples were homogenized using a handheld homogenizer in RIPA lysis buffer (Beyotime Biotechnology, Jiangsu, China) consisting of 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and multiple inhibitors. The homogenate was incubated on ice for 20 minutes. The lysate was then centrifuged at 12 000g for 20 minutes at 4°C and the protein concentrations were then measured using an enhanced Bicinchoninic Acid (BCA) protein assay kit (Beyotime Biotechnology). Equal amounts of protein (20 μg) were resolved using 12% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a Hybond polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). The membrane was blocked in 5% non-fat milk for 1 hour at room temperature and then incubated with the appropriate primary antibodies (occludin, ZO-1, GLP-1, Akt, p-Akt, Bcl-2 and Bax) at 4°C overnight. The product information and dilutions used of each antibody are as follows: occludin (1:1000, Novus Biologicals, #NBP1-87402), ZO-1 (1:500, Santa Cruz Biotechnology, #sc-10804), GLP-1R (1:1000, Santa Cruz Biotechnology, #sc-66911), p-Akt (phosphor-S473, 1:1000, Bioworld Technology, #BS4006, Louis Park, MN, USA), Akt (1:1000, Bioworld Technology, #BS1810), Bcl-2 (1:1000, Bioworld Technology, #BS1511), Bax (1:1000, Abcam, #ab32503) β-actin (1:1000, Bioworld Technology, #AP0731) and GAPDH (1:1000, Bioworld Technology, #AP0063). The membrane was washed in Tris-buffered saline (TBS) with 0.1% Tween-20 and incubated with the appropriate HRP-conjugated secondary antibody for 1 hour at room temperature. The membrane was then visualized with an enhanced chemiluminescence system (Thermo Scientific, Rockford, IL, USA). β-actin and GAPDH were used as loading controls.

2.10 | ELISA assay

At 14 days post-trauma, the colon samples were collected and proteins were extracted by homogenizing in ice-cold RIPA lysis buffer with a mix of the protease inhibitor PMSF. Homogenates were centrifuged at 12 000g for 20 minutes. The supernatant was obtained and total protein levels were determined with a BCA kit. The level of IL-6 in the colon was measured using a commercially available ELISA kit (RayBiotech, Norcross, GA, USA). A standard curve was constructed using a serial dilution of IL-6 standards and then used to calculate IL-6 concentrations of the test samples. Values are expressed as pg/mL. Blood samples for measurement of serum β-lactate were taken immediately before euthanasia and the serum was transferred to polyethylene tubes and stored at −80°C until analysis. The level of serum β-lactate was assayed by ELISA kit according to the manufacturer’s instructions (RayBiotech, Norcross, GA, USA). Values are expressed as μg/mL.

2.11 | Statistical analysis

Data of the NSS values were analyzed by two-way analysis of variance (treatment × time, ANOVA) with repeated measures followed
by the Bonferroni test. All other data were analyzed by one-way ANOVA. All data are presented as the mean ± standard error of the mean (SEM). The significant difference threshold was set at \( P = .05 \). Statistical analysis was performed using SPSS statistics V19.0 software.

3 | RESULTS

3.1 | Cb treatment improved neurological deficits in TBI mice

NSS was evaluated at 1, 4, 24 hour, 4, 7, 10, and 14 days after brain trauma. The results are shown in Figure 2. There was a significant increase in NSS among all the groups subjected to brain trauma, except the sham group. NSS was significantly decreased in the Cb-treated group compared to the TBI group at 4 days (\( P < .01 \)), 7 days (\( P < .01 \)), 10 days (\( P < .01 \)), and 14 days (\( P < .05 \)) post-trauma, suggesting that Cb treatment effectively alleviated neurological deficits in TBI mice.

3.2 | Cb treatment ameliorated brain edema in TBI mice

Brain edema was measured by brain water content at 24 hour post-trauma. As shown in Figure 3A, brain water content increased in TBI mice compared to that of the sham group (\( P < .05 \)), while Cb treatment led to a significant decrease in brain water content (\( P < .05 \)).

3.3 | Cb treatment delayed neuronal degeneration in TBI mice

The results are shown in Figure 3B. Fluoro-Jade C-positive cells (FJC+) labeling of neurons in the sham group was non-existent. Fluoro-Jade C-positive cells (FJC+) were increased in the cortical injury area and in an adjacent location in the TBI group. In contrast, the number of FJC+ cells in Cb-treated mice was significantly less than in the TBI group.

As shown in Figure 4, the neurons in the TBI group were loose in arrangement and some of them were absent, but in the Cb treatment group, the Nissl’s bodies increased. In brief, Cb treatment attenuated the pathologic changes and neuronal loss in TBI mice.

3.4 | Cb treatment ameliorated BBB impairment in TBI mice

Representative images and quantitative evaluation of EB extravasations in three groups are shown in Figure 5A-E. The amount of EB dye in the TBI group (\( P < .01 \)) was significantly increased compared to that in the sham group, indicating brain barrier impairment. However, the Cb group showed a significant decrease in EB extravasations (\( P < .01 \)) compared to the TBI group.

The fluorescence signal intensity from occludin and ZO-1 labeling largely appeared between adjacent endothelial cells in the sham group (Figure 6A-C). By contrast, in the TBI group, this intensity was dramatically lower than in the sham group.

This effect was confirmed by WB, which revealed that levels of occludin and ZO-1 in the TBI group were markedly higher compared to the sham group. Cb treatment significantly increased expression of the occludin and ZO-1 proteins (occludin: \( P < .01 \) and ZO-1: \( P < .01 \), Figure 6D-F).

3.5 | Cb treatment ameliorated neuronal apoptosis in TBI mice

We measured the expression of apoptosis-related protein markers at 14 days post-trauma. Compared with the sham group, the ratio of p-Akt/Akt was significantly reduced in the TBI group (\( P < .001 \), Figure 7A and B). In contrast, the ratio of p-Akt/Akt in the Cb-treated mice was significantly higher when compared with the TBI group (\( P < .05 \), Figure 7A and B), suggesting that Cb treatment could activate Akt. Bcl-2 expression was lower in the TBI group than in the sham group 24 hour after TBI but was elevated after Cb treatment (Figure 7C and D). Bax levels were elevated in the TBI group and were reduced after treatment with Cb (Figure 5C and D). The ratio of Bcl-2/Bax was significantly lower in the TBI group compared with the sham group (\( P < .001 \), Figure 7C and D). These data demonstrate that Cb activated the PI3K/Akt pathway and inhibited neuronal apoptosis in TBI mice.

3.6 | Cb treatment decreased the colonic IL-6 level and ameliorated gut barrier impairment

Inflammatory cytokines exacerbate GI damage after TBI. The level of colonic IL-6 in TBI mice was significantly higher than the sham group, while Cb treatment resulted in a significant decrease (\( P < .05 \), Figure 8A). In addition, lactate levels in the TBI group were significantly higher compared with the sham group (\( P < .01 \), Figure 8B). Treatment with Cb resulted in lower lactate levels than in the TBI group (\( P < .05 \), Figure 8B).
**FIGURE 3** *Clostridium butyricum* treatment reduced brain edema and attenuated neuronal degeneration at 24 hour after TBI. (A) Brain edema, determined with brain water content and calculated by dry/wet ratio. Error bars indicate SEM; *P < .05 vs sham group and #P < .05 vs TBI group; n = 4-5 per group. (B) Representative images of the Fluoro-Jade C staining in different groups at 24 hour after TBI. Cells with green staining are defined as degenerating neurons. (a) Representative images of the Fluoro-Jade C staining in sham group, (b) Representative images of the Fluoro-Jade C staining in TBI group, (c) Representative images of the Fluoro-Jade C staining in Cb group, and (d) Quantitative analyses of Fluoro-Jade C-positive cells in three groups. The arrows indicated Fluoro-Jade C-positive cells; Magnification 200×. Scale bar = 100 μm. n = 3 per group; **P < .01 versus sham group. ##P < .01 versus TBI group.

**FIGURE 4** Representative images of Nissl’s staining. Scale bars = 100 μm.
The level of GLP-1R was measured by western blot at 14 days post-trauma. The level of GLP-1R in the brain was remarkably decreased in the TBI group compared with the sham group ($P < .01$, Figure 9A and B), whereas Cb treatment ($P < .01$, Figure 9A and B) resulted in a significant increase, suggesting that Cb treatment could activate cerebral GLP-1R protein in the TBI mice.

4 | DISCUSSION

A number of studies have shown that TBI are associated with profound changes in gut dysfunction. Our previous studies have confirmed that Cb is effective in the treatment of brain disorders, by virtue of producing a large amount of butyrate. These effects of Cb can be mainly explained by its ability to increase the secretion of gut hormones GLP-1. However, mechanistic insights are lacking, especially regarding how probiotics-derived GLP-1 pathways to attenuate TBI-induced brain barrier impairment and neuronal damage. Our results demonstrate that Cb treatment effectively improved neurological deficits, brain edema, and BBB impairment in TBI mice. Cb treatment could lead to a decrease in neuronal apoptosis and to the amelioration of colonic inflammation and gut barrier impairment. Cb treatment increased the levels of GLP-1 in the colon, and GLP-1 receptor protein expression in the brain. This was correlated with an elevated level of the gut hormone GLP-1, which confers beneficial metabolic effects and protects against TBI in mice.

Neurological deficits are often the cause of disability after TBI. Our experiment used a mouse model in which TBI was induced with the weight-drop method, which frequently resulted in cortical contusion and a series of neurological impairments. In our previous studies, Cb treatment was shown to potentially attenuate cognitive disorder in mice. Consistent with our present study, Cb treatment significantly ameliorated TBI-induced neurological deficits. At the same time, morphological changes in the lesioned brain were likely to be parallel to alterations in behavior. Neuronal death is a cause of clinical deterioration and poor outcome in TBI. Focal cortical injury is associated with the delayed death of neurons, likely due to retrograde damage of cortical projections. We analyzed Fluoro-Jade C positive (FJC+) cells, which are used as a reliable indicator of degenerating neurons, after TBI. There was an effect of Cb treatment when administered to FJC+ cells after injury in mice.

Cerebral edema peaks within the first 24-72 hour after TBI and is considered a primary causal factor of neuronal injury and neurological deficits after TBI. In this study, Cb treatment decreased the water content in the 24 hour period after trauma. This indicates that Cb prevents brain endothelial barrier dysfunction and suggests that the reduction in brain water content is at least partially due to reduced vasogenic edema. Although the mechanisms that cause post-TBI brain edema have not been fully elucidated, disruption of the BBB is considered a major cause of brain edema and subsequent brain injury.

The BBB, a physical barrier separating brain extracellular fluid, plays an important role in maintaining the homeostasis of the brain.

3.7 | Cb increased the levels of GLP-1 in the colon and upregulated GLP-1R expression in the brain

As shown in Figure 9C, the level of GLP-1 protein was measured by immunohistochemistry. The TBI group had a lower level of GLP-1 than the sham group. However, the level of colonic GLP-1 in mice treated with Cb was higher than in the TBI group.
BBB-related TJ proteins, which could maintain BBB integrity, include transmembrane (claudins and occludin) and cytoplasmic proteins (ZO-1, ZO-2, and ZO-3). The loss and degradation of which are closely related to an increase in BBB permeability. BBB impairment can result in fluid amassing and a subsequent increase in intracranial pressure, which can further evoke brain edema and neuronal death. Increases in TJ protein levels are beneficial for the integrity of the BBB. Protection against BBB-integrity damages induced by TBI is probably achieved through the recovery of TJ proteins. In this study, Cb treatment improved BBB impairment induced by TBI in mice.

Thus, it is likely that the post-TBI reductions in BBB permeability and brain edema demonstrated in the present study are related to the anti-apoptosis properties of Cb. Neuronal apoptosis, which is coincident with the development of TBI, plays an important role in brain injury pathogenesis and may represent a hopeful target for treatment. The Akt pathway, a central mediator regulating neuronal growth and survival metabolism, has been shown to be involved in the pathogenesis of neuron death and neurological outcome after cerebral contusion in mice. Our results showed that C. butyricum treatment could activate Akt in TBI mice. The activation of Akt can regulate downstream targets, such as Bcl-2 and Bax, which play fundamental roles in the development of TBI. Our result showed that C. butyricum treatment significantly increased the level of Bcl-2 and decreased the level of Bax in TBI mice. Moreover, our previous study also found that Cb is able to exert neuroprotective effects against I/R injury mice through an increase of the Bcl-2/Bax ratio after C. butyricum pretreatment. Taken together, these results suggest that Cb treatment could exert an anti-apoptotic effect via the Akt pathway in TBI mice.
Gut microbiota dysfunction is linked to brain disorders. Accumulating evidence has shown that TBI is a common occurrence following GI dysfunction, along with the secretion of pro-inflammatory cytokines, degradation of intestinal TJ proteins, and increase in intestinal permeability. Previous studies have shown that SCFA-producing bacteria could alleviate inflammatory responses. In this study, Cb treatment ameliorated inflammation in TBI mice by decreasing the levels of IL-6. Intestinal permeability in TBI patients or animals always changed, as evaluated by the serum \( \delta \)-lactate level. \( \delta \)-lactate, a microbe-dependent metabolite, can pass through the disrupted intestinal barrier into the blood. In the present study, Cb treatment attenuated intestinal permeability in TBI mice by reducing the \( \delta \)-lactate level in the GI lumen. TJ proteins play a critical role in the intestinal barrier and directly regulate intestinal permeability. In the present study, Cb treatment ameliorated intestinal barrier impairment in the TBI mice. Taken together, this suggests that Cb treatment can attenuate intestinal dysfunction in TBI mice by inhibiting inflammation, decreasing the level of serum \( \delta \)-lactate and improving intestinal barrier impairment. The molecular mechanisms underlying how Cb attenuated neuronal impairment in the TBI mice requires further insight.

**FIGURE 7** *Clostridium butyricum* treatment reduced apoptosis cells and apoptosis-related proteins in the injured cortical area at 14 days after TBI. (A and C) Representative western blots showed expression of p-Akt, Akt, Bcl-2 and Bax. (B and D) Quantitative analyses of the ratio of p-Akt/Akt and Bcl-2/Bax from each group. \( \beta \)-actin was the loading control. Data are normalized to the sham group. Error bars indicate SEM; **P < .01 vs sham group. \#P < .05 vs TBI group; \( n = 4-6 \) per group.

**FIGURE 8** *Clostridium butyricum* treatment alleviated intestinal barrier dysfunction at 14 days after TBI. (A) Level of IL-6 in the colon. (B) Intestinal permeability assay, measuring \( \delta \)-lactate concentration in serum. (C) Representative Western blots showed colon expression of Occludin relative to GAPDH. (D) Quantitative analyses of Occludin from each group. GAPDH was the loading control. \( n = 5-6 \) per group. (E) Immunofluorescence staining for colonic Occludin. Representative images for \( n = 3 \). Magnification 200\( \times \). Scale bar = 100 \( \mu \)m. Error bars indicate SEM; **P < .01 vs sham group. \#P < .05 vs TBI group.
The gut-brain axis is defined as a two-way regulative pattern between the gut and brain. Brain-gut peptide, such as GLP-1, might exert neuroprotective effects via gut-brain axis. The levels of SCFAs in the gastrointestinal tract are influenced by the microbial composition. The level of SCFAs in the GI may change along with the occurrence of intestinal dysbiosis. Cb treatment could produce abundant butyrate from the fermentation of fibers and result in increased butyrate levels in the colon. Furthermore, butyrate could bind to the G-protein-coupled receptors GPR41 and GPR43 and stimulate gut hormone production in the colon and associated microbial activity with gut L-cells. GLP-1 is widely expressed in the brain, specifically throughout the cerebrum, including the cerebral cortex, hippocampus, and substantia nigra. Activated GLP-1R can exert multifarious neuroprotective effects in Alzheimer’s, Parkinson’s diseases and stroke by combining with GLP-1 or long-acting GLP-1R agonists. Consistent with studies showing that GLP-1 can stabilize the...
integrity of the BBB, activating GLP-1R may be one of the mechanisms by which Cb attenuated BBB impairment in TBI mice. Our results showed that Cb treatment could exert neuroprotective activity, which may be via stimulating secretion of GLP-1 and subsequently activating GLP-1R in the brain. Our hypothesis regarding this neuroprotective mechanism is presented in Figure 10. However, there are also some limitations. Neuropeptide and metabolic parameters other than butyrate and GLP-1 were not measured, so we are not able to say which one changed initially and whether or not the Cb treatment caused these changes. A further study to investigate this potential mechanism is necessary.

In conclusion, our findings demonstrated the neuroprotective effect of Clostridium butyricum in TBI mice and the involved mechanisms were partially attributed to the elevating GLP-1 secretion through the gut-brain axis. Our current studies possibly offer an intriguing opportunity of treatment of TBI patients.

CONFLICT OF INTERESTS
The authors declare that there is no conflict of interests regarding the publication of this paper.

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**SUPPORTING INFORMATION**

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