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Clostridium butyricum Strains Suppress Experimental Acute Pancreatitis by Maintaining Intestinal Homeostasis

*Li-Long Pan, Wenying Niu, Xin Fang, Wenjie Liang, Hongli Li, Wei Chen, Hao Zhang, Madhav Bhatia, and Jia Sun**

Scope: Acute pancreatitis (AP) is a common abdominal inflammatory disease. Disturbed gut homeostasis secondary to pancreatic inflammation aggravates the condition retroactively. The current study investigates potential beneficial effects of *Clostridium butyricum (C. butyricum)* strains on AP and underlying mechanisms.

Methods and results: *C. butyricum* strains MIYAIRI 588 (CBM588) and CGMCC0313.1 (CB0313.1) were supplemented to mice for three weeks before experimental AP or SAP induction. Both CBM588 and CB0313.1 protected against AP, as evidenced by reduced serum amylase and lipase levels, pancreatic edema, and myeloperoxidase activity. Amelioration of both experimental AP and SAP by CB0313.1 indicated a non-model-specific effect. Moreover, *C. butyricum* inhibited pancreatic neutrophil and dendritic cell infiltration, nucleotide-binding domain leucine-rich repeat-containing family, pyrin domain-containing 3 inflammasome activation, and pro-inflammatory pathways. Additionally in the gut, *C. butyricum* strains attenuated AP-associated intestinal inflammation and barrier dysfunction, accompanied with reduced pathogenic bacteria *Escherichia coli* and *Enterococcus* penetration into pancreas. Gut microbiome analyses further revealed that beneficial effects of *C. butyricum* on pancreatic-gut homeostasis were correlated with improved dysbiosis. In particular, relative abundance of *Desulfovibrionaceae* decreased, and *Verrucomicrobiaceae Clostridiaceae* and *Lactobacillaceae* increased.

Conclusions: For the first time, a protective effect of *C. butyricum* in AP by modulating intestinal homeostasis is demonstrated.

1. Introduction

Acute pancreatitis (AP) is a common clinical inflammatory disorder, for which effective therapeutic or nutritional intervention remains elusive and highly needed. Pathogenesis of AP involves premature activation of proenzymes, pancreatic autodigestion and, acinar cell damage, which is clinically marked by increased serum amylase and lipase levels. Immune cell influx following pancreatic damage potentiates the local inflammatory response. In severe cases when local inflammation is not properly controlled, these pathological events reinforce each other, leading to uncontrolled systemic inflammatory responses affecting multiple remote organs, which closely correlate with AP-associated mortality. Innate immune cells including polymorphonuclear neutrophils, macrophages, and dendritic cells with distinct cell-surface and intracellular markers are key players during the acute phase of the disease. Activation of these cells has been closely associated with the development and severity of acute inflammatory conditions.[1–4]

Intestinal homeostasis has been shown to play a pivotal role in modulating the development of AP. Intestinal dysfunction is frequently associated with AP.[5–7] Changes in gut permeability/motility lead to bacterial translocation and activation of gut-associated

L.-L. Pan
School of Medicine
Jiangnan University
Wuxi 214122, P. R. China

W. Niu, X. Fang, Dr. W. Liang, Dr. H. Li, Prof. W. Chen, Prof. H. Zhang, Prof. J. Sun
State Key Laboratory of Food Science and Technology
Jiangnan University
Wuxi 214122, P. R. China
E-mail: jiasun@jiangnan.edu.cn

W. Niu, X. Fang, Dr. W. Liang, Dr. H. Li, Prof. W. Chen, Prof. H. Zhang, Prof. J. Sun
School of Food Science and Technology
Jiangnan University
Wuxi 214122, P. R. China

Prof. M. Bhatia
Inflammation Research Group, Department of Pathology
University of Otago
Christchurch 8140, New Zealand

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lymphoid tissues that contribute to systemic complications of AP. Gut dysbiosis is associated with intestinal barrier dysfunction and high mortality rates observed in patients with severe AP (SAP).[8] Higher levels of *Enterococcus* and lower levels of Bifidobacterium were found in AP patients.[7] Thus, preventing AP-associated intestinal dysfunction might be the key for effective therapy.

An efficient nutraceutical approach to prevent intestinal barrier disruption and modulate gut microbiome balance is by intervening with immunomodulatory probiotics. *Clostridium butyricum* belongs to short chain fatty acids (SCFA)-producing probiotics and possess immunomodulatory properties.[9,10] Earlier studies have shown that particular *C. butyricum* strains suppressed colitis via IL-10 production[9] and alleviated ovalbumin-induced allergic airway inflammation and food allergy in mice.[11,12] We have earlier shown that *C. butyricum* protects against autoimmune diabetes by modulating intestinal immune homeostasis and inducing pancreatic regulatory T cells.[13] However, the effects of *C. butyricum* strains on experimental AP have not been reported. The current study explored the efficacy of *C. butyricum* strains as potential beneficial probiotics in AP and underlying mechanisms.

2. Experimental Section

2.1. Animals

8-week BALB/c and C57BL/6J mice (Su Pu Si Biotechnology Co., Ltd., Suzhou, Jiangsu, China) were used in this study. Mice were bred at the Animal Housing Unit of Jiangnan University (Wuxi, Jiangsu, China) under 23–25 °C and 12 h light–dark cycle with unlimited access to food and water. All mice were allowed to acclimatize to the laboratory conditions over the course of 1 week prior to the experiments. All experimental procedures were approved by the animal ethics committee of Jiangnan University (JN.No20160927-20161022[65]) and were performed in accordance with the European Community guidelines (Directive 2010/63/EU).

2.2. Induction of AP and Pretreatment with *C. butyricum*

BALB/c mice (20 ± 2 g) were randomly assigned to four groups ($n = 7$): control (CON) group, caerulein (CAE) group, CBM588+CAE group, and CB0313.1+CAE group. The mice received hourly intraperitoneal injections with normal saline or saline containing CAE (50 μg kg^{-1}, Sigma-Aldrich, MO, USA) for 12 h to induce AP.[14] To examine the biological effects of *C. butyricum*, mice were treated with two specific *C. butyricum* strains CBM588 (9.6×10^8 CFU per kg per day) and CB0313.1 (5.7×10^9 CFU per kg per day) by gavage once a day for 21 consecutive days before induction of AP. Both CBM588 (Miyarisan Pharmaceutical, Tokyo, Japan) and CB0313.1 (Qingdao East Sea Pharmaceutical Co. Ltd., Shangdong, China) are spore-forming probiotics and they were suspended in sterile PBS before being administrated to mice. Mice in CON group and CAE group were given sterile PBS only as a control. 1 h after the last injection, mice were anesthetized with sodium pentobarbital and tissues were removed. A SAP model was induced by 12 hourly CAE intraperitoneal injections followed immediately by an injection of lipopolysaccharide (LPS; 20 mg kg^{-1}) after last cearulein injection.[15] In this model, C57BL/6J mice (25 ± 2 g) were randomly divided into five groups ($n = 7$): CON group, CAE+LPS group, CBM588+CAE+LPS group, CB0313.1+CAE+LPS group, and CB0313.1(L)+CAE+LPS group. The methods of pretreatment with *C. butyricum* were same as AP models. The dose of CB0313.1 (L)+CAE+LPS group is 9.6×10^8 CFU per kg per day, which is the same as that for the CBM588+CAE+LPS group. Choice of mouse strains for the two models was based on standard protocols of model induction. In CAE induced mild edematous AP, BALB/c mice are more sensitive than C57BL/6[16,17]; while in SAP which may cause undesired mortality during model induction, C57BL/6J is preferably used.[15,18] 3 h after the last injection, mice were anesthetized with sodium pentobarbital and tissues were removed.

2.3. Serum Amylase and Lipase Activity

Harvested blood was centrifuged at 1600 × *g* for 10 min after coagulating at room temperate for 25 min. The supernatant (serum) was then collected and stored at −80 °C until analysis. Serum amylase and lipase activity were determined by using serum amylase assay kit (Jian Cheng Bioengineering Institute, Nanjing, China) and serum lipase assay kit (Jian Cheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol.

2.4. Pancreatic Edema and Myeloperoxidase Activity

A portion of freshly harvested pancreatic tissue was trimmed and weighted. Pancreatic water content was evaluated by the ratio of initial weight (wet weight) of the pancreas to its weight after incubation at 80 °C for 48 h (dry weight).[19] Myeloperoxidase (MPO) activity was measured by using MPO assay kit according to the manufacturer's protocol (Jian Cheng Bioengineering Institute, Nanjing, China).

2.5. Inflammatory Cytokine Measurement

Harvested samples of pancreas and colon were homogenized in 20 mM phosphate buffer (pH 7.4) and centrifuged at 10 000 × *g* for 15 min at 4 °C. The supernatants were used for measuring tissue levels of inflammatory mediators. Serum prepared by centrifugation of harvested blood samples were used for measuring systemic levels of inflammatory mediators. Tissue or systemic levels of tumor necrosis factor alpha (TNF-α), IL-6, MCP-1, and IL-12 were determined with the enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the instructions of manufacturer. Results were expressed as pg ug^{-1} of DNA (for pancreas), pg mg^{-1} (for colons), pg mL^{-1} (for serum). DNA assay was performed fluorometrically by using H33258 (Yeasen, Shanghai, China) by the method of

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Labarca and Paigan[20] and calf thymus DNA (Yeasen, Shanghai, China) as standard.

2.6. Histological Examination

Freshly harvested pancreatic and colonic samples were fixed with 4% paraformaldehyde, dehydrated in ethanol, and then embedded with paraffin. Prepared sections (5 μm) were stained with haematoxylin and eosin using standardized protocols. Morphological changes of pancreas and colon were examined under a DM2000 light microscope (Leica Microsystems GmbH, Heidelberg, BW, Germany) at 40× magnification. Pancreatic injury was evaluated based on edema, inflammatory cell infiltration, hemorrhage, and necrosis.[21]

2.7. Western Blotting

Pancreatic and colon tissues were homogenized in RIPA buffer (ThermoFisher Scientific, MA, USA) with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, MO, USA). Equal amounts of total proteins were separated via polyacrylamide SDS-PAGE gel and transferred onto PVDF membrane and probed with following antibodies: nucleotide-binding domain leucine-rich repeat containing family, pyrin domain-containing 3 (NLRP3), phosphor (p)-nuclear factor κ-B (NF-κB) p65, p-extracellular signal-regulated kinase 1/2 (ERK), total-ERK, p-c-Jun N-terminal kinase 1/2 (JNK), total-JNK (all 1:1000, Cell Signaling Technology, Boston, MA, USA), caspase-1 p20, interleukin-1β (IL-1β), ZO-2, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (all 1:500,Santa Cruz Biotechnology, Santa Cruz, CA, USA), toll-like receptor 4 (TLR4) (Proteintech, Rosemont, PA, USA), IL-18 (Abcam, Cambridge, MA, USA), ZO-1, Occludin (Life technology, Carlsbad, CA, USA). Blots were developed by enhanced chemiluminescence (PerkinElmer, Waltham, MA, USA).

2.8. SCFA Analysis

Fecal samples were collected on the day before induction of AP and then stored at −80 °C until use. Concentrations of acetate, propionate, and butyrate in fecal samples were analyzed by GC-MS (Shimadzu, Kyoto, Japan) as previously described.[22] Briefly, feces (50 mg) were first homogenized in 500 μL of saturated NaCl solution. Thereafter, fecal samples were acidified with 40 μL 10% sulfuric acid. Next, 800 μL diethyl were added to the samples to extracted SCFA. Samples were then centrifuged at 14 000 rpm for 15 min at 4 °C, and supernatants was used for analysis with GC-MS.

2.9. DNA Extraction and qPCR for Pathogenic Bacteria and *C. butyricum*

Fecal samples were collected after the last CAE injection in two models and then stored in −80 °C until use. Microbial genomic DNA was extracted from thawed feces and pancreatic samples using Fast DNA Spin Kit for Soil (MP Biomedicals, California, CA, USA) following manufacturer's instructions. SYBR Green PCR reagents (Yeasen, Shanghai, China) were used to determine the relative expression of pathogenic bacteria and *C. butyricum*, which were normalized by Universal DNA. Primer sequences are as follows: *Escherichia coli*: Forward: 5′-CATGCCGCGTGTATGAAGAA-3′ and Reverse: 5′-CGGGTAACGTCAATGAGCAAA-3′; *Enterococcus*: Forward: 5′-CCCTTATTGTTAGTTGCCATCATT-3′ and Reverse: 5′-ACTCGTT

GTACTTCCCATTGT-3′; *C. butyricum*: Forward: 5′-CCTCCT TTCTATGGAGAAATCTAGCA-3′ and Reverse: 5′-TGTAGCTT GACCTTTTTAAGTTTTGA-3′; Universal: Forward: 5′-TCCTA CGGGAGGCAGCAGT-3′ and Reverse: 5′-GACTACCAGGGT ATCTAA TCCTGTT-3′.

2.10. Analysis of Fecal Microbiota Composition

DNA extracted from stool samples of SAP models were used to analyze microbiome at Illumina MiSeq system. The V4 region of 16S rRNA was amplified using primers (sense: 5′-AYTGGGYDTAAAGNG-3′; antisense: 5′-TACNVGGG TATCTAATCC-3′), then purified and quantified by using Gene Clean Turbo (MP Biomedicals, Santa Ana, CA, USA) and Quant-iT PicoGreen dsDNA Assay Kit (Life Technology, Carlsbad, CA, USA). The libraries were prepared using TruSeq DNA LT Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequenced for 500+7 cycles on Illumina MiSeq by using the MiSeq Reagent Kit (500 cycles-PE).

2.11. Flow Cytometry

Freshly harvested pancreatic tissues were cut into small pieces. After being digested in 0.75 mg mL^{-1} collagenase-P (Boehringer Mannheim, Ingelheim, R P, Germany) at 37 °C for 15 min, the digested pancreatic pieces were dissociated with gentleMACS Dissociator (Miltenyi Biotecnology, Bergisch Gladbach, NRW, Germany), then filtered through 75 μm filters with PBS/10%FBS immediately. The single cell suspension of pancreas was centrifuged at 300 g for 5min and then washed with PBS several times. Single cell suspensions were stained for 30 min at 4 °C with several monoclonal antibodies. For determining neutrophils and dendritic cells, cells were surface stained with anti-CD45 (Miltenyi Biotecnology, Bergisch Gladbach, NRW, Germany), -CD11b, -Ly6G, and -CD11c (BioLegend, San Diego, CA, USA). For macrophages, cells were fixed and permeabilized by employed Cell Fixation & Permeabilization Kit (FcMACS, NanJing, China) after being surface stained with anti-CD45, -CD11b, -F4/80 (BioLegend, San Diego, CA, USA), and subsequently stained with anti-CD206 (BioLegend, San Diego, CA, USA). Stained cells were analyzed on Attune NxT (Thermo Fisher Scientific, Waltham, MA, USA).

2.12. Statistical Analysis

All data are expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance followed by Dunnett's test post hoc test using GraphPad Prism (version 5; GraphPad

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Software Inc., San Francisco, CA, USA). *p* value less than 0.05 was considered as a statistically significant difference.

3. Results

3.1. *C. butyricum* Supplementation Alleviates Experimental AP

The effects of *C. butyricum* were examined in CAE-induced experimental AP. As shown in **Figure 1**, pretreatment with CB0313.1 for 3 weeks reduced the severity of AP, evidencing by reduced serum amylase, lipase levels as well as pancreatic edema and MPO activity (Figure 1A–D). Microscopic morphological examination confirmed the protective effect of CB0313.1 as marked by improved cellular morphology, reduced pancreatic edema, inflammatory cell infiltration, and acinar necrosis (Figure 1E). Consistent with a reduced pancreatic inflammation, CB0313.1 pretreatment significantly reduced serum and pancreatic cytokine production including TNF-α, IL-6, and IL-12 (Figure 1F,G). In comparison, CBM588 supplementation reduced pancreatic edema, serum lipase and cytokines and pancreatic TNF-α, but demonstrated moderate effects on other parameters (Figure 1A–G). Collectively, *C. butyricum* strains demonstrate modulatory effects on experimental AP.

3.2. *C. butyricum* Attenuates SAP

To exclude a model-specific effect and as AP-associated mortality mostly arises from SAP,[23] we next examined the effects of *C. butyricum* on experimental SAP induced by CAE together with LPS. Consistently, CB0313.1 supplementation markedly attenuated SAP-mediated pancreatic injury, evidenced by decreased serum amylase, lipase levels as well as pancreatic edema and MPO

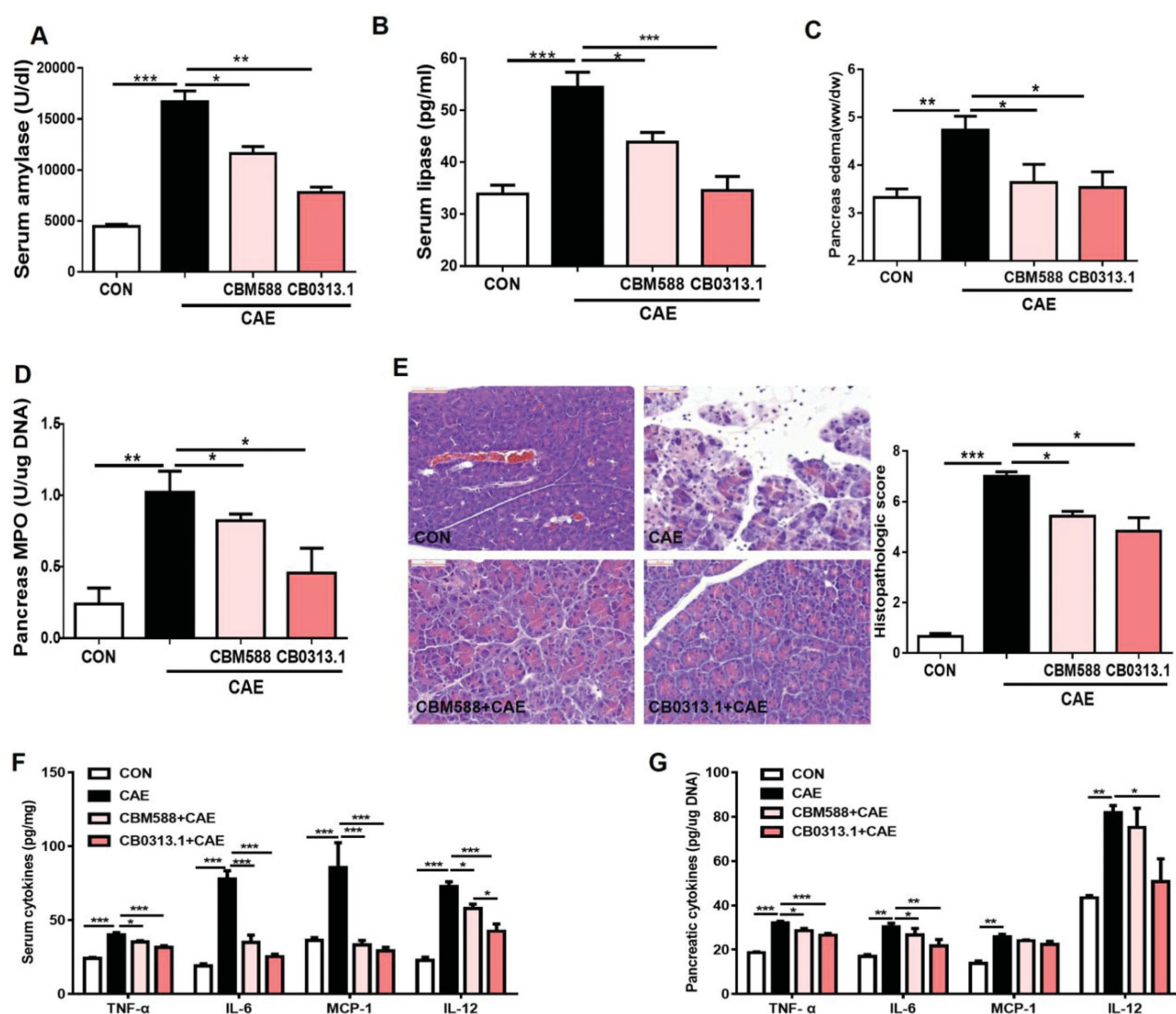

Figure 1. *C. butyricum* supplementation alleviates experimental AP. BALB/c mice were pretreated with CBM588 or CB0313.1 per os for 3 weeks. AP was then induced by caerulein. A) Serum amylase, B) serum lipase, C) pancreatic edema, and D) pancreatic MPO levels. E) Representative haematoxylin and eosin-stained pancreatic sections and histologic score; scale bars, 50 μm. F) Serum TNF-α, IL-6, MCP-1, and IL-12 levels. G) Pancreatic TNF-α, IL-6, MCP-1, and IL-12 levels. Data were shown as mean ± SEM ($n = 7$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. MPO: myeloperoxidase.

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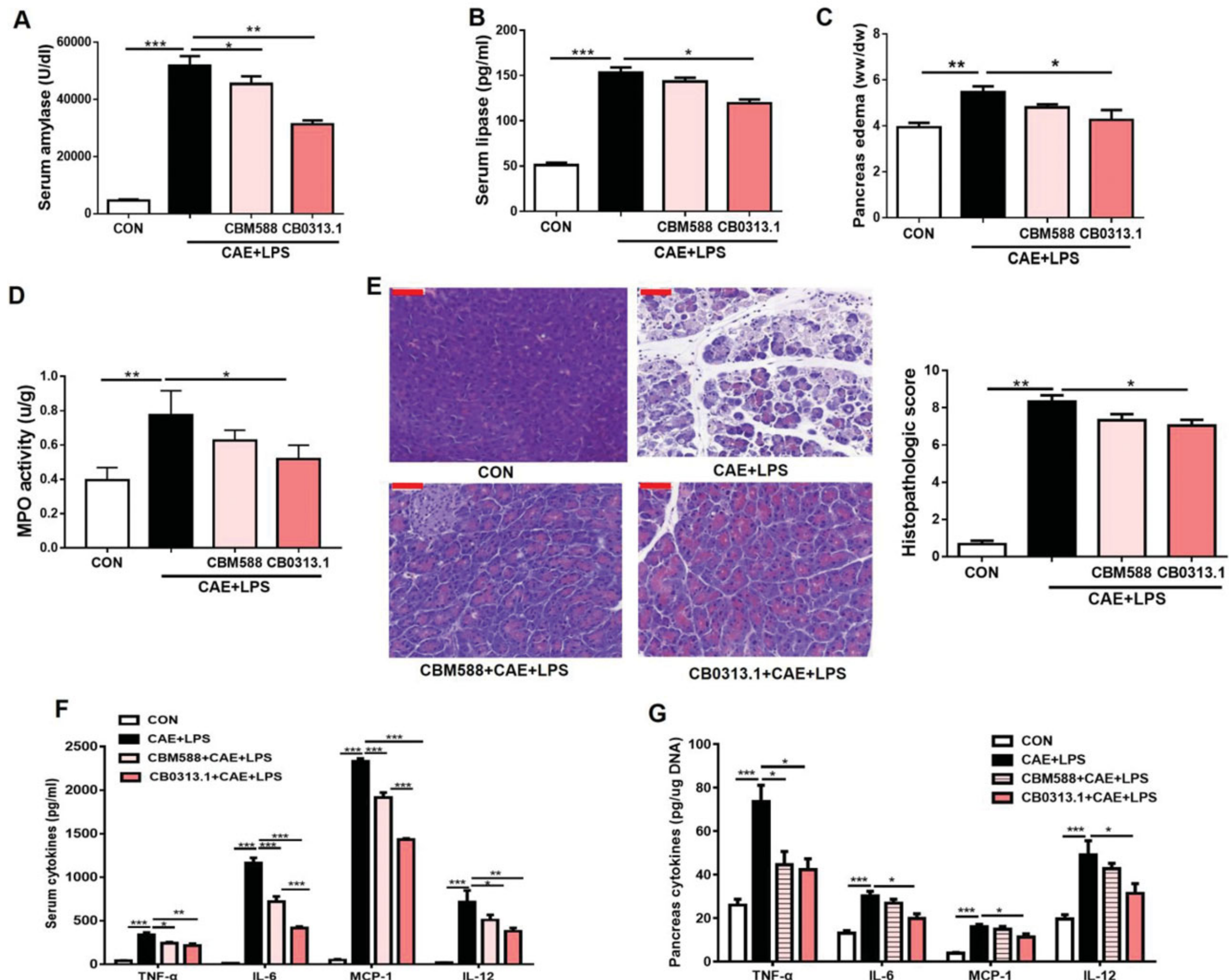

Figure 2. *C. butyricum* CB0313.1 supplementation attenuates experimental SAP. C57BL/6J mice were pretreated with CBM588 or CB0313.1 for 3 weeks and underwent induction of SAP. A) Serum amylase, B) serum lipase, C) pancreatic edema, and D) pancreatic MPO levels. E) Representative haematoxylin and eosin-stained pancreatic sections and histologic score; scale bars, 50 μm. F) Serum TNF-α, IL-6, MCP-1, and IL-12 levels. G) Pancreatic TNF-α, IL-6, MCP-1, and IL-12 levels. Data were shown as mean $\pm$ SEM ($n = 7$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

activity (**Figure 2**A–D). These results were further confirmed by microscopic pancreatic morphological examination (Figure 2E). Consistent with reduced severity of SAP, CB0313.1 administration significantly lowered systemic and pancreatic cytokine (TNF-α, IL-6, MCP-1, and IL-12) production (Figure 2F,G). CBM588 treatment did not exert an overall protective effect on pancreatic injury during SAP, although serum pro-inflammatory cytokine markers were reduced (Figure 2A–G).

3.3. *C. butyricum* Modulates Pancreatic Infiltration of Neutrophils and Dendritic Cells

As shown above, *C. butyricum* strains reduced pancreatic MPO activity, which is a neutrophil marker. Indeed, innate immune cells are important players in the progression of AP.[24] Thus, we subsequently analyzed the effects of *C. butyricum* on innate immune cell recruitment into the pancreas. Consistent with decreased pancreatic inflammatory responses, both CBM588 and CB0313.1 supplementation profoundly attenuated the infiltration of $CD11b^+Ly6G^+$ neutrophils (**Figure 3**A) and $CD45^+CD11c^+$ dendritic cells (Figure 3B). In contrast, AP-associated macrophage accumulation or polarization did not seem to be affected with CBM588 or CB0313.1 supplementation (Figure S1, Supporting Information).

3.4. *C. butyricum* Suppresses AP-Mediated Pancreatic NLRP3 Inflammasome Activation

Activation of NLRP3 inflammasome is documented in the pathogenesis of AP.[25–28] We then investigated whether *C. butyricum* exerted their effects by modulating NLRP3 inflammasome activation. Induction of AP conspicuously activated NLRP3 inflammasome. Consistent with their protective effects, both CBM588 and CB0313.1 attenuated AP-mediated activation of NLRP3 inflammasome, evidencing by decreased NLRP3, caspase-1 p20, cleaved IL-1β, and cleaved IL-18 expression (**Figure 4**A). Bacterial translocation and penetration importantly contribute to local and systemic complications associated with AP.[28,29] TLR4

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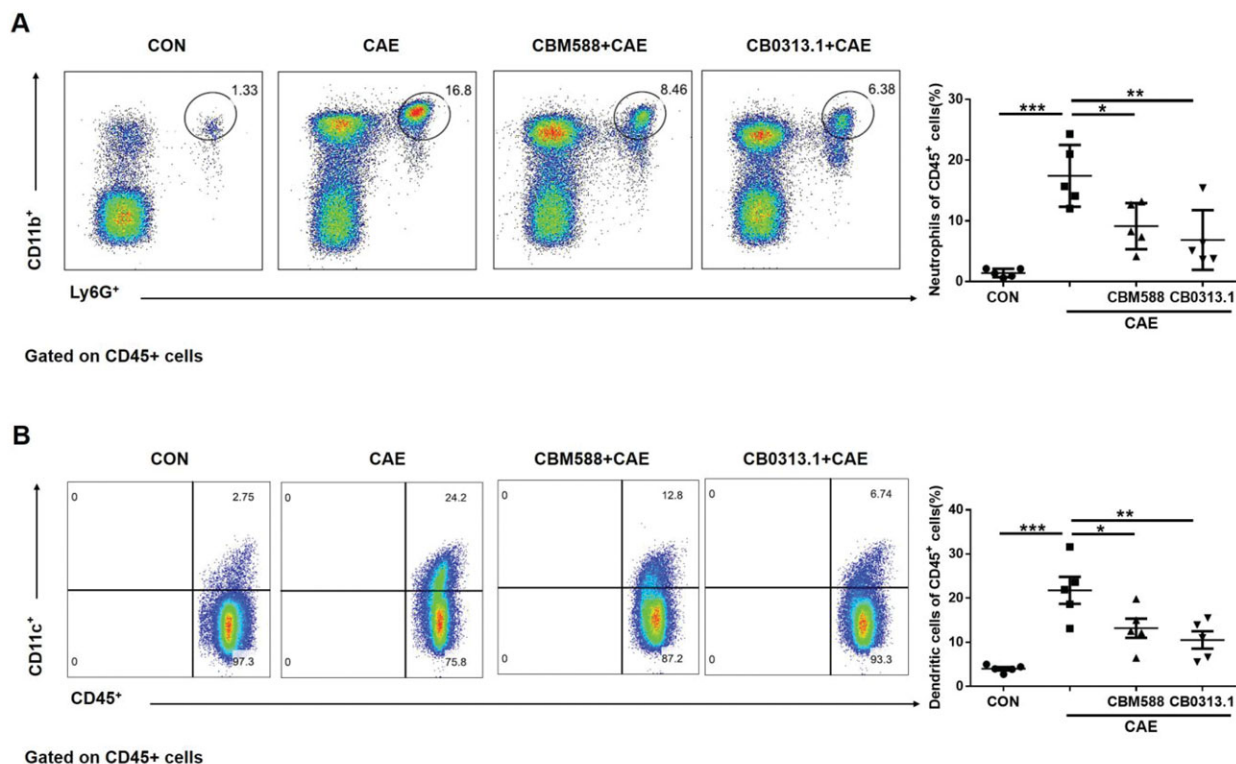

Figure 3. *C. butyricum* modulates pancreatic infiltration of neutrophils and dendritic cells BALB/c mice were pretreated with CBM588 or CB0313.1 for 3 weeks, then AP was induced by caerulein. A) Frequency of $CD11b^+Ly6G^+$ neutrophils (gated on $CD45^+$ cells). B) Frequency of $CD45^+CD11c^+$ dendritic cells (gated on $CD45^+$ cells). Data were shown as mean ± SEM ($n = 7$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

together with NLRP3 inflammasome activation play a critical role in the propagation of AP.[30] Both CB0313.1 and CBM588 administration resulted in downregulation of AP-induced TLR4 expression. Additionally, we found that *C. butyricum* suppressed AP-mediated phosphorylation of ERK and JNK as well as the NF-κB p65 subunit at Ser536, a key step in TLR4 mediated NF-κB activation,[30,31] thereby suppressing TLR4/mitogen-activated protein kinase (MAPK)/NF-κB p65 activation (Figure 4B).

3.5. *C. butyricum* Attenuates AP or SAP-Associated Intestinal Inflammation

Intestinal dysfunction accompanies AP and the resultant increase in intestinal permeability may cause bacterial translocation to complicate the conditions.[29] CB0313.1 supplementation markedly attenuated AP-induced increase of colonic TNF-α, IL-6, and IL-12 levels, while TNF-α and IL-6 levels in CBM588 supplemented group showed only trends of decrease (**Figure 5**A). Further notably, both CB0313.1 and CBM588 administration attenuated AP-mediated colonic NLRP3 inflammasome activation (Figure 5B). In SAP, both CBM588 and CB0313.1 supplementation significantly reduced colonic TNF-α, IL-6, and IL-12 levels. However, CB0313.1 but not CBM588 alleviated AP-mediated colonic MCP-1 production (Figure 5C). Furthermore, both CB0313.1 and CBM588 downregulated SAP-induced TLR4 expression (Figure 5D). Downstream of TLR4, *C. butyricum* suppressed AP-mediated phosphorylation of ERK, JNK, and NF-κB p65 (Ser536), thereby suppressing the inflammatory responses in colon (Figure 5E–G).

3.6. *C. butyricum* Attenuates Intestinal Barrier Dysfunction and Permeability during SAP

We next evaluated whether *C. butyricum* strains attenuated intestinal barrier dysfunction and permeability and the consequences in experimental SAP model. Tight junction proteins (TJPs) are important indexes to characterize intestinal barrier function and reduced TJP expression leads to increased intestinal permeability.[32] Therefore, expression of major TJPs (ZO-1, ZO-2, and occludin) in colon was subsequently measured. As shown in **Figure 6**A, both CB0313.1 and CBM588 pretreatment restored SAP-induced downregulation of ZO-1, ZO-2, and occludin. The protective effect on intestinal barrier integrity was further confirmed by histological examination, evidencing by increased crypt length in colon of *C. butyricum*-supplemented mice (Figure 6B). Bacterial translocation across the gut barrier is a direct consequence of the increased intestinal permeability. *E. coli* and *Enterococcus* are the frequent pathogens causing pancreatic infection and systemic complications during SAP.[28] Indeed, CB0313.1 pretreatment was associated with decreased relative abundance of *E. coli* and *Enterococcus* in pancreas (Figure 6C), consistent with strengthened barrier function. CBM588

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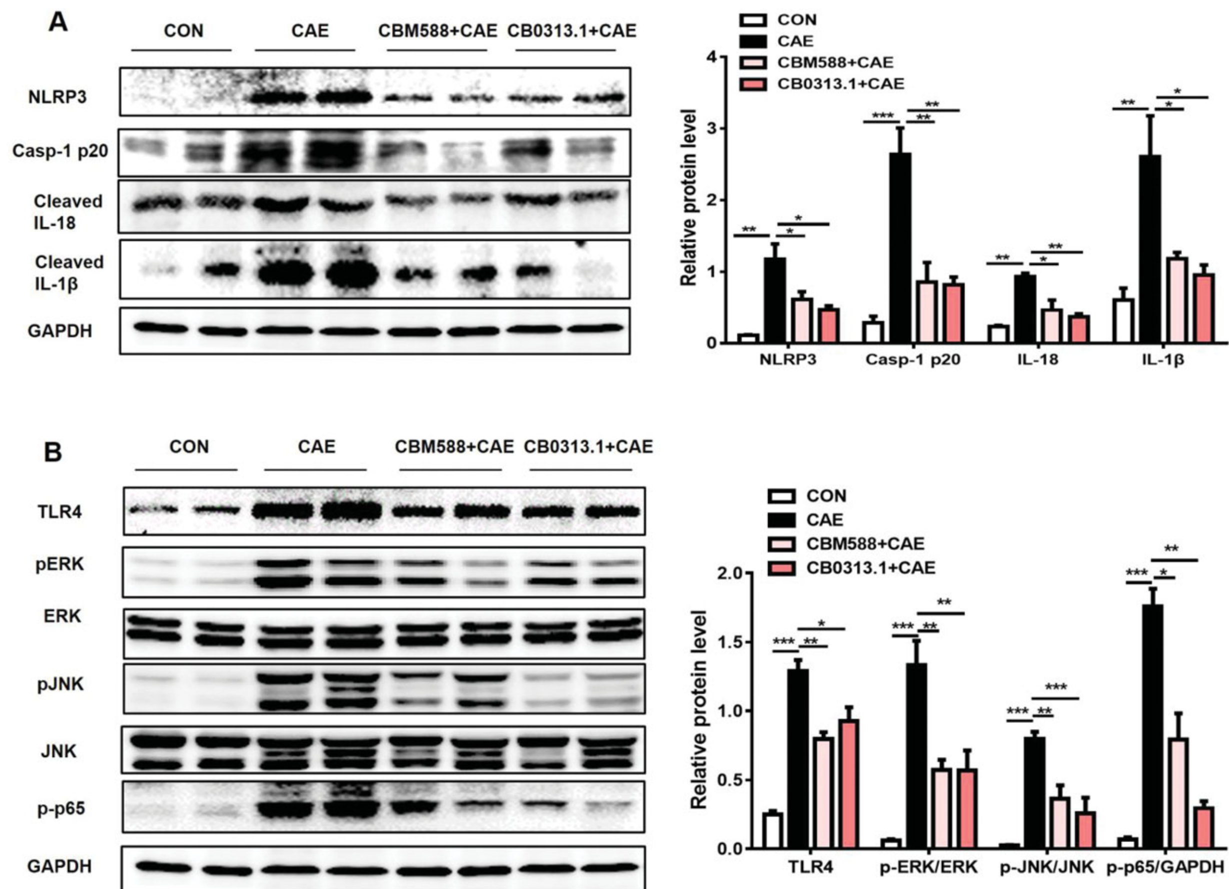

Figure 4. *C. butyricum* attenuates AP-mediated activation of pancreatic NLRP3 inflammasome and TLR4 signaling pathway BALB/c mice were pretreated with CBM588 or CB0313.1 for 3 weeks and underwent induction of AP. A) Western blot and quantitative analysis of NLRP3, caspase-1 p20, cleaved IL-18, and cleaved IL-1β in pancreas. B) Western blot and quantitative analysis of TLR4, p-ERK, ERK, p-JNK, JNK, and p-NF-κB p65 in pancreas. GAPDH was used as loading control. Data were shown as mean $\pm$ SEM ($n = 7$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

supplementation only caused reduced pancreatic *Enterococcus*, but a trend toward decrease of *E. coli* (Figure 6C). These data demonstrated that *C. butyricum*, especially CB0313.1 supplementation strengthened intestinal barrier function and decreased bacterial translocation during SAP. SCFA produced by gut microbiota have anti-inflammatory properties and protect gut barrier function and integrity, thereby contributing to gut homeostasis.[33] CB0313.1 and CBM588 supplementation markedly increased total SCFA levels in feces of mice before SAP induction and CB0313.1 seemed to have stronger impact than CBM588 on SCFA production (Figure S2, Supporting Information). In comparison, after SAP induction, SCFA levels were found significantly increased in CB0313.1- but not CBM588-treated mice, suggesting that CB0313.1 could partially restore SCFA levels in mice with SAP (Figure 6D).

3.7. Modulatory Effects of *C. butyricum* on Gut Microbiota in SAP Mice

Gut dysbiosis was correlated with the progression of SAP in patients.[7] We have earlier shown that *C. butyricum* can restore type 1 diabetes-induced gut dysbiosis.[10] Thus finally, we examined the effects of *C. butyricum* on SAP-induced perturbation of intestinal microbiota composition. Principal component analysis (PCA) showed that the gut microbiota communities in mice pretreated with *C. butyricum* were markedly different from the untreated group (**Figure 7**A). Diversity of gut microbiota in SAP mice was significantly reduced as evidenced by lower shannon indexes and higher simpson indexes compared to the control group (Figure 7B). *C. butyricum* supplementation further decreased the microbiota diversity (Figure 7B). Analysis of the microbiota at various taxonomic levels indicated that notable alterations to the composition of gut microbiota occurred in two *C. butyricum* strains-treated groups (Figure 7C,D). In particular, at the family level, Desulfovibrionaceae and Rikenellaceae were significantly expanded in SAP mice. CBM588 of note, remarkably restored the relative abundance of Desulfovibrionaceae (Figure 7E). Further analysis demonstrated that taxonomic groups Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Akkermansia, and *Akkermansia muciniphila* were consistently and significantly increased in mice after *C. butyricum* treatment (Figure 7C,E and Figure S3A, Supporting Information). Intriguingly, the Clostridiaceae and Lactobacillaceae with profound anti-inflammatory properties[34,35] were significantly more abundant in CB0313.1-supplemented mice (Figure 7E and

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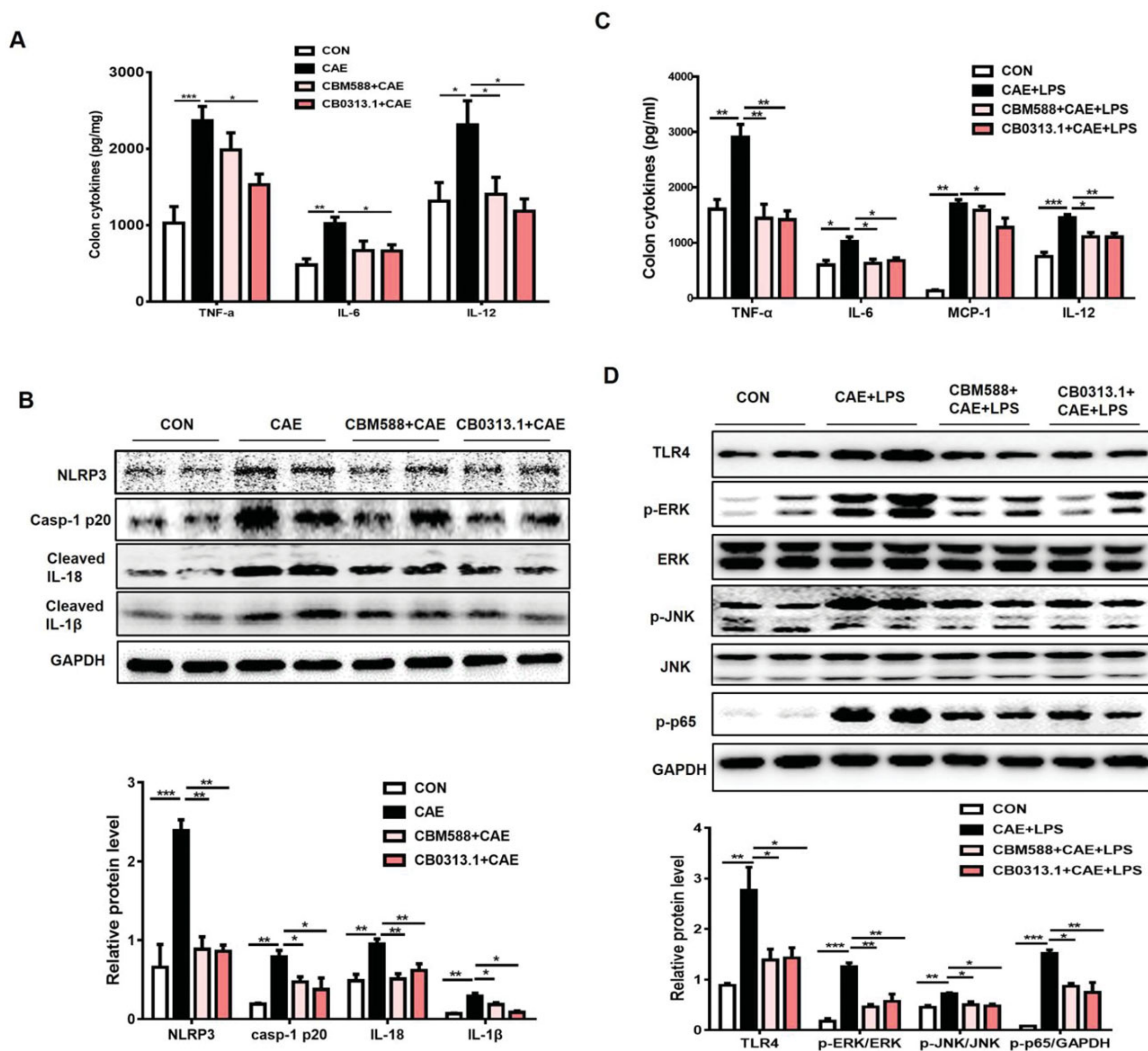

Figure 5. *C. butyricum* strains alleviates AP or SAP-associated intestinal injury. BALB/c mice were pretreated with CBM588 or CB0313.1 for 3 weeks and underwent induction of AP. A) Colonic TNF-α, IL-6, and IL-12 levels. B) Western blot and quantitative analysis of colonic NLRP3, caspase-1 p20, cleaved IL-18, and cleaved IL-1β. C57BL/6J mice were pretreated with CBM588 or CB0313.1 for 3 weeks and underwent induction of SAP. C) Colonic TNF-α, IL-6, MCP-1, and IL-12 levels. D) Western blot and quantitative analysis of colonic TLR4, p-ERK, ERK, p-JNK, JNK, and p-NF-κB p65. GAPDH was used as loading control. Data were shown as mean ± SEM ($n = 7$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure S3B, Supporting Information). In addition, Lachnospiraceae, Clostridiales, and Prevotellaceae decreased in SAP mice but were not restored with *C. butyricum* pretreatment except that CBM588 enriched the Prevotellaceae (Figure S3C, Supporting Information).

4. Discussion

In the present study, we demonstrate that supplementing *C. butyricum* stains, alleviates experimental AP and SAP. The protective effect of *C. butyricum* was partly mediated by inducing intestinal homeostasis, as evidenced by ameliorated intestinal permeability and reshaped gut microbiota. Improved intestinal homeostasis results in suppressed activation of NLRP3 inflammasome and TLR4 signaling pathway in pancreas and reduced pancreatic infiltration of neutrophils and dendritic cells.

Changes in intestinal motility and microbiome, immune response, and mucosal barrier function during AP may lead to bacterial translocation and subsequent pancreatic necrosis infection, which is one of the principal causes of AP-associated death.[36] Probiotics, for their capabilities to strengthen gut integrity and prevent bacterial translocation have been explored as a promising nutritional intervention strategy for AP.[6,37,38] However currently, clinical efficacious probiotic therapy is still lacking. Conflicting evidence has been reported regarding the role of probiotics in regulating AP in experimental and clinical settings. For example, it has been demonstrated in rodent models that administration

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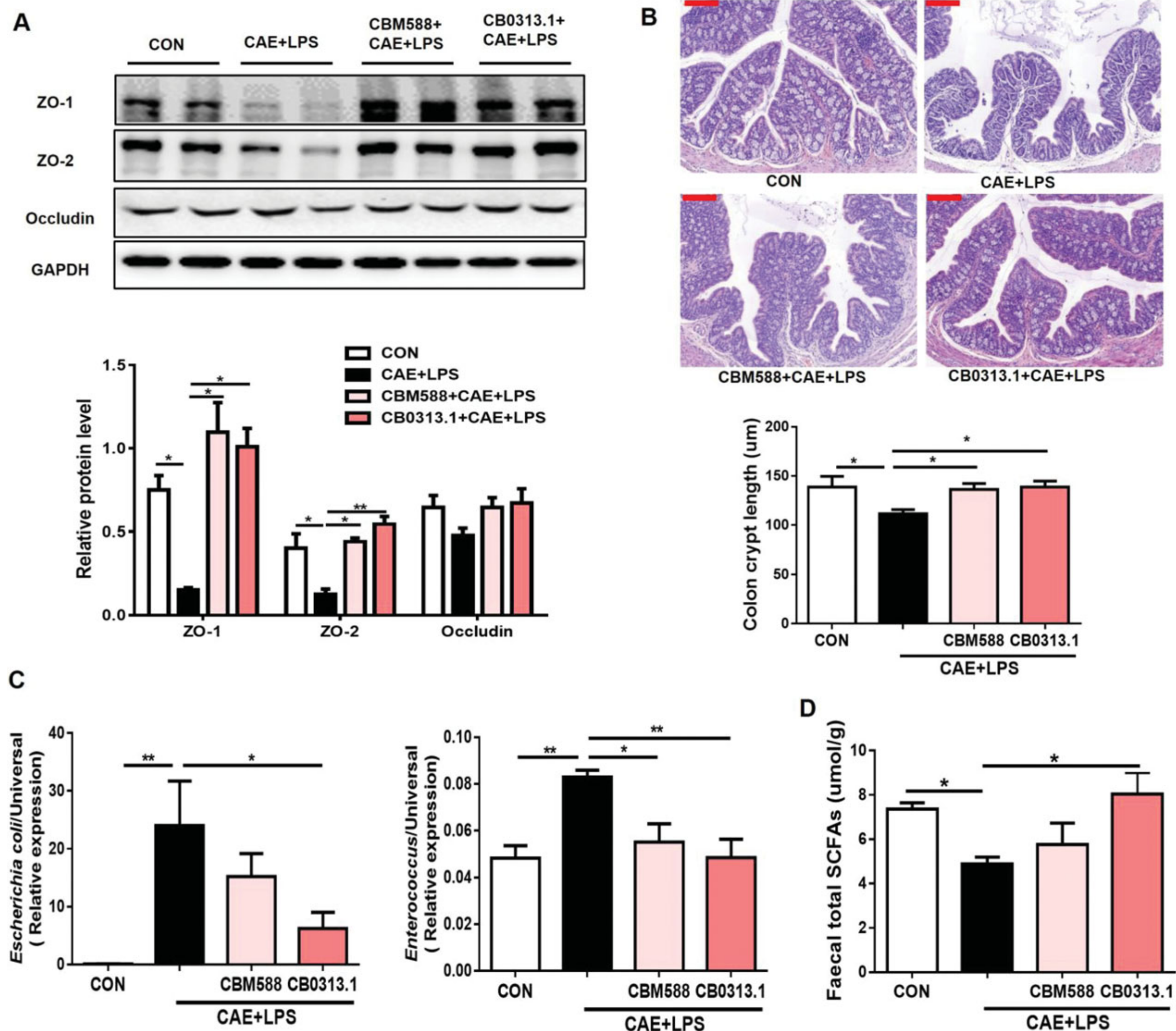

Figure 6. *C. butyricum* alleviates intestinal barrier dysfunction during SAP. C57BL/6J mice were pretreated with CBM588 or CB0313.1 for 3 weeks and underwent induction of SAP. A) Western blot and quantitative analysis of colonic ZO-1, ZO-2, and Occludin. B) Representative haematoxylin and eosin-stained colonic sections and quantification of crypt length; scale bars, 100 μm. C) Relative abundance of *Escherichia coli* and *Enterococcus* in pancreas. The day before the induction of SAP, fecal samples were collected and D) Faecal total SCFA levels were determined. Data were shown as mean ± SEM ($n = 7$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. SCFAs: short-chain fatty acid.

of either *Saccharomyces boulardii* alone[39] or mixtures of different probiotics[40,41] afforded protective effects on AP. In contrast, others reported a pro-inflammatory role of probiotic mixture.[42] In clinical settings, probiotic prophylaxis in SAP has been contraindicated. Qin et al. demonstrated that *Lactobacillus plantarum* enteral feeding reduced disease severity, improved intestinal permeability and clinical outcomes.[43] However, in PROPATRIA, a multicenter, randomized, double-blind, placebo-controlled trial with 200 patients with predicted SAP, multispecies probiotic prophylaxis did not reduce the risk of infectious complications and was associated with an increased risk of mortality.[44,45] Following studies involving multispecies probiotic supplementation with EN early abandoned after PROPATRIA study but seemed to support the results that no significant trend was identified for an effect on gut permeability or endotoxemia in AP,[46,47] although a positive effect was observed with reduced endotoxin levels.[47] In view of these findings, efficacies of current probiotic formulations are still insufficient in AP and new probiotics are needed. *C. butyricum* stains, CBM588 and CB0313.1, are commercial probiotics to treat patients with gut dysbiosis, diarrhea, and irritable bowel syndrome.[48] They are able to promote the beneficial value of intestinal bacteria, maintain intestinal barrier integrity, and regulate mucosal immunity.[9,49] It has been demonstrated that CBM588 alleviated acute experimental colitis[9] and high-fat diet-induced non-alcoholic fatty liver disease.[50] We have earlier shown that CB0313.1 improves type 1 diabetes and obesity.[13,22] Herein, we extended beneficial effects of *C. butyricum* to experimental AP. To the best of our knowledge, this is the first report to demonstrate the efficacy of *C. butyricum* in experimental AP.

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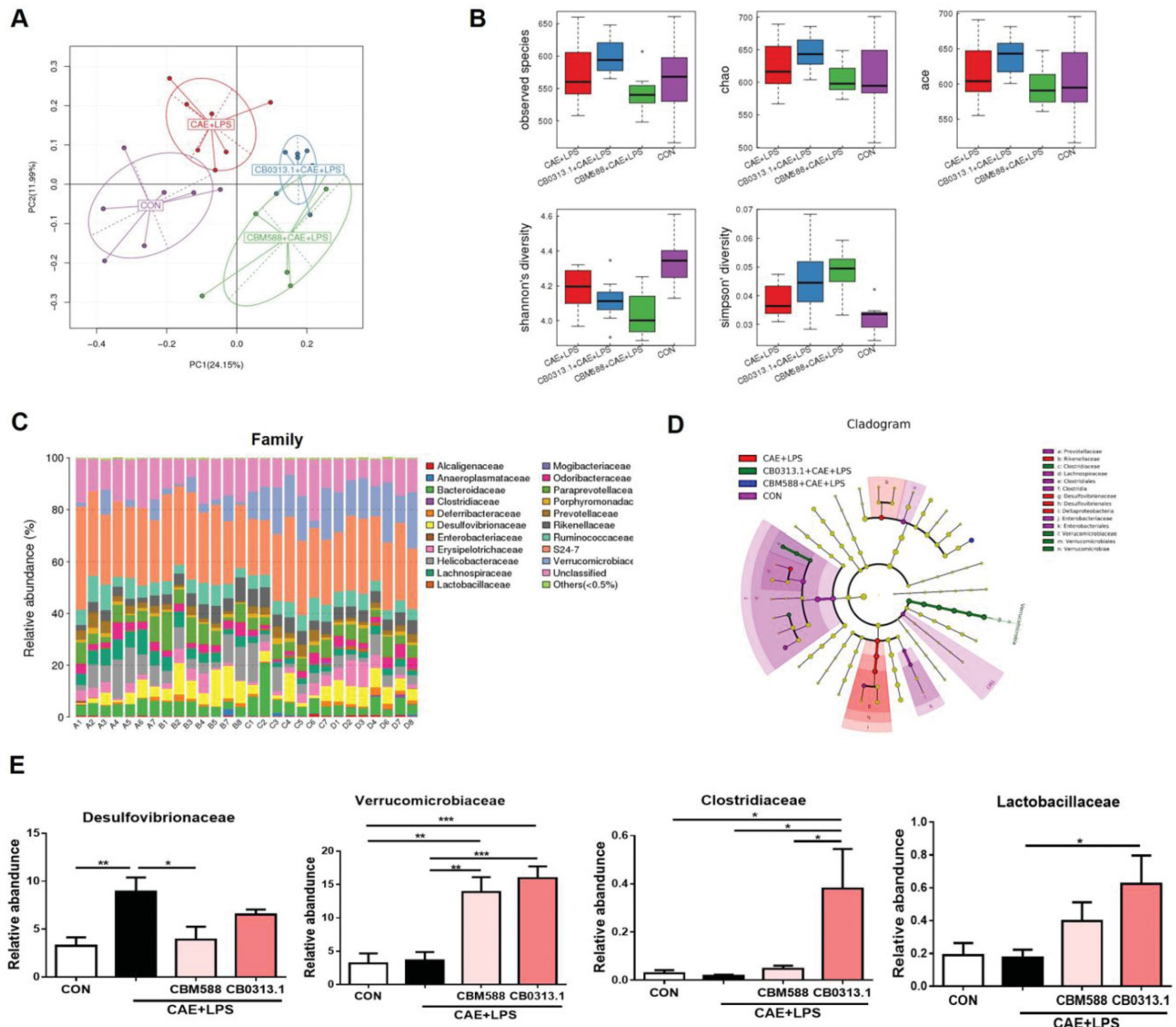

Figure 7. Modulatory effects of *C. butyricum* on gut microbiota in SAP mice. C57BL/6J mice were pretreated with CBM588 or CB0313.1 for 3 weeks and underwent induction of SAP. A) Principal component analysis (PCA) based on OTU abundance. B) α-diversity analysis between four groups. Shannon diversity index and Simpson index were calculated using the OTU table in QIIME. C) The taxonomic composition distribution among four groups of family-level. D) Cladograms generated by LEfSe indicating differences in taxa between four groups. E) Relative abundance of Desulfovibrionaceae, Verrucomicrobiotaceae, Clostridiaceae, *and* Lactobacillaceae were shown (family-level). Data were shown as mean ± SEM ($n = 7$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Compromised intestinal barrier function and resultant increased intestinal permeability have been associated with the development of SAP, causing bacterial and endotoxin translocation to propagate pancreatic and systemic inflammation leading to multiple organ injury.[5,19,51] Preserving intestinal barrier function and integrity represents a strategic approach to reduce morbidity associated with AP and benefits recovery.[19] Indeed, the rationale for using probiotics to prevent or limit intestinal damage during the course of AP resides in their beneficial effects on gut barrier function.[52] As shown here, *C. butyricum* intervention restored intestinal barrier function and subsequently reduced the dissemination of pathogenic bacteria (*E. coli* and *Enterococcus*) into pancreas, thereby alleviating second attack of inflammatory events in pancreas and systemically.

Meanwhile, the levels of SCFAs in feces were increased in both *C. butyricum* supplemented groups. SCFAs are important gut microbiota metabolites that have demonstrated multiple salubrious effects, including maintaining intestinal immune homeostasis and protecting intestinal barrier.[53–57] Moreover, their effects are not always restricted to the intestinal tract but may enter the circulation and influence cells of peripheral tissues.[58,59] Indeed, by GC-MS, we observed that the amount of SCFA, and particularly of butyrate, in the feces of *C. butyricum*-supplemented mice were higher than in the untreated control mice. Consequently, the protective effects of *C. butyricum* on AP and associated intestinal injury observed are attributable at least in part to the production of its SCFAs metabolites.

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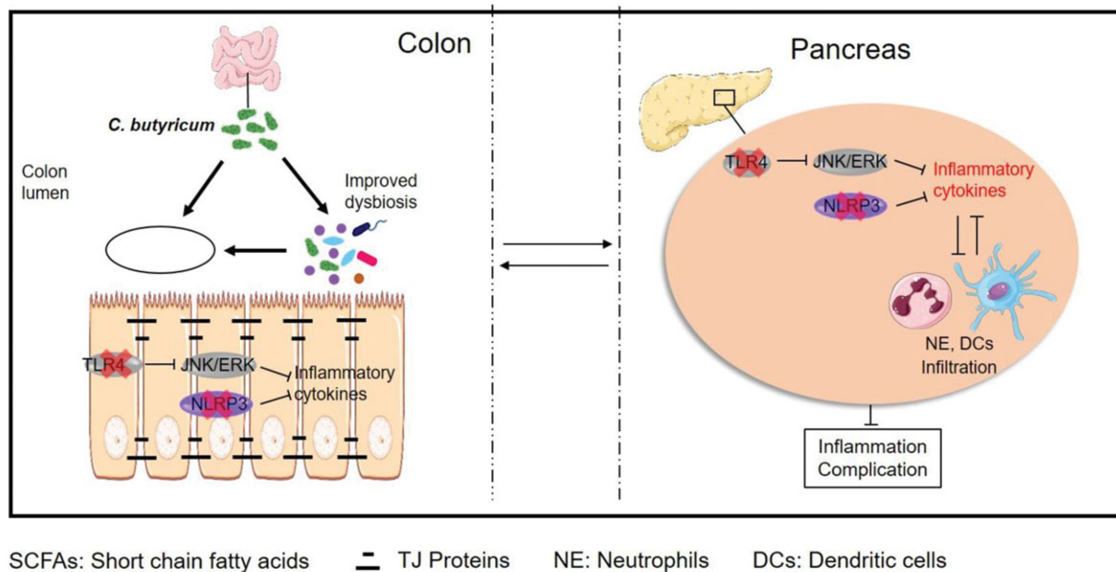

Figure 8. A proposed model depicting the mechanism by which *C. butyricum* protect against AP and associated intestinal dysfunction. *C. butyricum* maintain gut immune and barrier homeostasis to shape pancreatic immune environment, thereby mitigating the development of AP.

Translocation of bacteria and endotoxins due to SAP-associated increased intestinal permeability is key to causing pancreatic necrosis and further aggravation of inflammation.[60] Innate immune signaling in response to bacterial components or products plays an important part in AP. TLR4 is a receptor for LPS derived from bacteria and they can also be activated by endogenous ligands, such as pancreatic elastase released during cell destruction. Activation of TLR4 results in transcriptional up-regulation of procytokines and NLPR3 inflammasome components,[61] which are all strong determinants of inflammation and organ damage in acute injury of the intestine and pancreas.[23,61] Consistently, we confirmed that TLR4 signaling was activated in pancreas and intestine in experimental AP and SAP and demonstrated that *C. butyricum* supplementation could suppress TLR4 mediated pro-inflammatory responses and NLRP3 inflammasome activation via down regulation of the NF-κB pathway. These findings suggest that *C. butyricum* ameliorated AP-mediated pancreatic injury in part through modulation of TLR4-related signaling pathway.

Taxonomic and functional alterations in intestinal microbiota have been described and characterized in different types of pancreatitis with or without comorbidity, including AP.[8,62,63] Under pathological conditions, locally altered environment in the gut may favor growth of specific bacterial taxa, leading to increased abundance of some of potential opportunistic pathogens and decreased abundance of some beneficial bacterial taxa. The shift in composition of intestinal microbiota in turn influenced the inflammatory environment in the gut.[64] Desulfovibrionaceae, which is one of the pro-inflammation/pathogenic bacteria, is associated with development of obesity, adipose tissue, and systemic inflammation.[65] *C. butyricum* induced decrease in Desulfovibrionaceae may therefore exert anti-inflammatory effects. It should be noted that taxonomic groups Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Akkermansia, and *A. muciniphila* increased consistently in two *C. butyricum* supplemented groups (Figure S3A, Supporting Information), indicating that *C. butyricum* promoted the establishment of a protective microbiota with enriched Akkermansia, which have been shown to have potential anti-inflammatory properties.[66,67] Both *Clostridiaceae* and *Lactobacillaceae* are potential probiotic members that alleviate inflammation and have an impact on gut barrier function.[22,68] It is particularly intriguing that only CB0313.1 treatment markedly increased those bacteria, suggesting that CB0313.1 may be more effective on colonization and thus beneficial to the growth of probiotics than CBM588. This may explain for better protective effects on SAP by CB0313.1 administration. Another notable observation is that *C. butyricum* treatment did not restore the impaired abundance of Lachnospiraceae and Clostridiales in SAP mice, which were reduced in acute necrotizing pancreatitis and Crohn's disease respectively.[13,69] The possible explanation is that domination of Verrucomicrobia suppresses the growth of these bacteria, which may also explain the decreased diversity of gut microbiota in *C. butyricum* treatment groups.

In summary, our data clearly demonstrate that *C. butyricum* protects experimental AP and related intestinal injury. The protective effects of *C. butyricum* as marked by decreased infiltration of neutrophils and dendritic cells in pancreas and inhibited inflammatory responses mediated via NLRP3 and TLR4 signaling pathways in pancreas and colon, mediated by maintaining gut homeostasis as evidenced by attenuated intestinal permeability and modulated gut microbiota. The current study provides the basis for future evaluation of *C. butyricum* as nutritional application in clinical AP.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Acknowledgements

L.-L.P. and W.N. contributed equally to this work. J.S. and L.-L.P. conceived the study project and designed the experiments. W.N. and L.-L.P. performed the experiments and analyzed the data, with general assistance from X.F., W.L., and H.L. W.C., H.Z., and M.B. provided intellectual inputs and contributed to data acquisition. W.N. and J.S. drafted the manuscript. All authors contributed to the interpretation of the experiments and critically reviewed the manuscript. All authors gave final approval of the work. The work was supported by funds from the National Natural Science Foundation of China (Grant Nos.: 81870439, 91642114, 31570915, and 81573420, National Youth 1000 Talents Plan), Jiangsu Province Recruitment Plan for High-level, Innovative, and Entrepreneurial Talents (Innovative Research Team), Jiangsu Province Qing Lan Project, the Fundamental Research Funds for the Central Universities (Grant Nos.: JUSRP51613A and JUSRP11866), National First-class Discipline Program of Food Science and Technology (Grant No: JUFSTR20180103) and Wuxi Social Development Funds for International Science & Technology Cooperation (Grant No: WX0303B010518180007PB).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

Clostridium butyricum, immunoregulation, intestinal homeostasis, pancreatic inflammation, gut microflora

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