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Caffeine as a microbiome modulator: a novel therapeutic strategy for hypertriglyceridemia-related acute pancreatitis

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ABSTRACT

Hypertriglyceridemia (HTG) is a common and significant contributor to acute pancreatitis (AP). Caffeine has been reported to have a protective effect against pancreatic disorders. However, the role of caffeine in hypertriglyceridemia acute pancreatitis (HAP) is still unknown. To investigate a new understanding of the relationship between caffeine and gut microbiota in HAP development. Using multi-omics analysis of 71 HAP and HTG patients, their characteristics of intestinal microecology were detected. HTG mice models were established through either pharmacological induction (P-407 intraperitoneal injection) or genetic ablation of *GPIHBP1* (*GPIHBP1*$^{-/-}$). Genes deferentially expressed in the gut were identified by RNA sequencing and the role of caffeine in reshaping gut microbial networks was investigated. Caffeine levels decreased significantly in HAP patients and were inversely correlated with the severity of HAP. Caffeine maintained the intestinal homeostasis and attenuated HAP through a gut microbiota-dependent manner in the mouse model. Specially, the commensal *Faecalibacterium prausnitzii*, which contributed most in distinguishing the differences between HTG and HAP patients, has been found to be related to the effect of caffeine on alleviating HAP. RNA sequencing combined with *TLR4*$^{-/-}$ mice revealed that TLR4/NLRP3 may be the key signaling pathway for caffeine to maintain intestinal homeostasis and alleviating HAP. This study reveals the gut metabolites and microbiota characteristics of HAP and HTG patients, and explores the therapeutic potential of caffeine against HAP from the perspective of gut microbiota.

1. Introduction

Acute pancreatitis (AP) remains a critical gastroenterological emergency, with hypertriglyceridemia (HTG) emerging as the third leading etiology responsible for 15%−20% cases[1-2]. Recent advances in gut-pancreas axis research have revealed microbial metabolites as key modulators of pancreatic inflammation, particularly short-chain fatty acids demonstrating protective effects in experimental AP models[3-4]. These discoveries highlight the therapeutic potential of targeting microbial-host metabolic crosstalk in pancreatic disorders.

Despite progress in hypertriglyceridemia acute pancreatitis (HAP) management, critical challenges persist across multiple dimensions. While pathogenic distinctions between HAP and other AP subtypes remain underexplored at the microbiome-metabolite interface, therapeutic development continues to stagnate due to an excessive focus on endogenous metabolites coupled with

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insufficient attention to dietary-derived modulators. Compounding these issues is the absence of translational models for evaluating microbiota-driven nutritional interventions. Moreover, a fundamental knowledge gap persists regarding the mechanistic connection between gut dysbiosis and HTG-specific pancreatic necrosis, which remains crucial for advancing clinical solutions.

Parallel developments in metabolic disease research provide crucial insights. Caffeine, a ubiquitous dietary component, demonstrates microbiome-dependent efficacy in obesity and diabetes through anti-inflammatory pathway modulation[5-7]. The success of fecal microbiota transplantation (FMT) in *Clostridioides difficile* infections offers methodological frameworks for establishing causal microbe-metabolite relationships[8-9]. These cross-disciplinary advances suggest untapped potential for dietary-microbiome engineering in pancreatic inflammation.

Building on existing gut-pancreas axis knowledge, we integrate nutritional science with microbial genomics to address HAP-specific therapeutic gaps. By adapting metagenomic mapping techniques from metabolic syndrome research and employing germ-free models from microbiome causality studies, we establish a novel experimental paradigm. This approach enables systematic investigation of caffeine's microbiota-mediated effects on HTG-driven pancreatic injury.

Herein, we combine longitudinal multi-omics profiling of HAP patients with mechanistic validation in gnotobiotic models. Our innovative three-phase methodology first identifies microbial-metabolomic signatures distinguishing HAP from HTG, then establishes causality through microbiota depletion/reconstitution experiments, ultimately pinpointing *Faecalibacterium prausnitzii* as the key caffeine-responsive symbiont. Scientifically, we discovered a microbial metabolic switch mechanism where caffeine reprograms gut communities to preferentially utilize anti-inflammatory pathways toll-like receptor 4 (TLR4)/NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) inhibition over pro-inflammatory endotoxin production. This breakthrough not only elucidates caffeine's paradoxical therapeutic efficacy in HAP but also establishes a new class of microbiome-targeted dietary therapeutics, fundamentally advancing our understanding of nutritional immunomodulation in organ-specific inflammation.

2. Materials and methods

2.1 Participants and sample collection

In January 2021 and January 2022, 71 patients from Shanghai General Hospital, including 36 HTG patients and 35 HAP patients, were randomly selected for the study. Eligible HTG groups were required to meet the criteria of having a serum triglyceride (TG) level greater than 11.3 mmol/L, or a fasting serum TG level between 5.65 and 11.3 mmol/L, or the presence of chylous serum[10]. AP was diagnosed according to the Revised Atlanta Classification[11]. The patients who simultaneously met the HTG criteria and AP diagnostic criteria were divided into HAP group. Patients on antibiotics, probiotics or laxatives within three months before recruited were excluded. Sample handling strictly followed established protocols[12-13]. Blood samples were collected from all patients. Pancreatitis patients' blood samples were collected within 24 h of the onset of their disease. The serum samples were obtained by centrifugation. All enrolled patients gave written consent. Our study was ratified by the Chinese Clinical Trial Registry (2300069239).

2.2 Metabolite extraction

For metabolite extraction, 50 mg of the stool samples from patients that have been collected and dried by filter paper was weighed, and 400 L of extraction solution with 0.02 mg/mL of *L*-2-chlorophenylalanine were added. The mixture was ground for 6 min at −10 °C, 50 Hz using the Wonbio-96c frozen tissue grinder (Shanghai Wanbo Biotechnology Co., Ltd., China), followed by ultrasonic extraction (40 kHz) at 5 °C for 30 min. LC-MS/MS analysis was performed on the supernatant after centrifuging the sample at 4 °C for 15 min. A Thermo UPLC-Q Exactive system equipped with an ACQUITY HSS T3 column (50 mm × 2.1 mm, 1.8 μm) was used for the LC-MS/MS analysis.

2.3 Metabolite data preprocessing and analysis

Data from raw LC/MS experiments were preprocessed using Progenesis QI software (Waters Corp., Milford, USA), and a three-dimensional data matrix in CSV format was exported. This three-dimensional matrix contained the following information: sample information, metabolite name, and mass spectral response intensity. Data peaks corresponding to internal standards, as well as those associated with false positives, have been removed, de-redundant, and merged. Metabolites were identified in parallel by HMDB Database, the Metlin, and the Majorbio Database.

Data analysis was conducted *via* Majorbio Cloud Platform. In any sample set, at least 80% of metabolic features were preserved. Then we normalized each metabolic trait by the sum of the minimum metabolite values. To reduce errors caused by sample preparation and instrument instability, we normalized the intensity of peaks in the mass spectrum and then we obtained the normalized data matrix. In addition, variables with relative standard deviation (RSD) > 30% of the quality control (QC) samples were removed, and lg processing was applied to obtain the final data matrix.

The variance analysis was performed based on the matrix file after data preprocessing. The R package "ropls" was used to conduct principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), as well as interactive validation over seven cycles assessing the stability of the model. Metabolites with variable importance in projection (VIP) $> 1, P < 0.05$ were determined to be significantly different metabolites based on VIP obtained by the OPLS-DA model and P value generated by Student's t-test.

Metabolic enrichment and pathway analysis were used to map differences in metabolites between two groups based on Kyoto Encyclopedia of Genes and Genomes (KEGG) data. Using enrichment analysis, we analyzed metabolites regardless of whether they were present or not in a function node. An annotation analysis of a single metabolite evolves into an annotation analysis of a group of metabolites.

2.4 Animal experiments

C57BL/6 mice (6−8 weeks, 20−22 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (China) and raised at room temperature of (23 ± 2) °C under specific pathogen free (SPF) conditions with 12-h light/dark cycles. *TLR4*$^{-/-}$ mice were purchased from Shanghai Model Organisms Laboratory (Shanghai, China). *GPIHBP1*$^{-/-}$ mice were sponsored by Professor Lu Guotao of the Medical School of Yangzhou University (China). We conducted animal experiments according to the instructions of the Institutional Animal Care and Use Committee (IACUC) at Shanghai General Hospital (2020AW095).

In the first series of core animal studies, we explored the effects of caffeine on HAP. A total of four groups of mice (n = 6) were randomly divided: HTG group (HTG), HTG with caffeine group (HTG + CAF), HAP group (HAP) and HAP with caffeine group (HAP + CAF). HTG models were established via injecting mice intraperitoneally with poloxamer 407 (P-407; 0.5 g/kg; HY-D1005, MedChem Express, China) on alternate time for 28 consecutive days[14]. The mice were intraperitoneally injected with caerulein (100 μg/kg; MedChem Express, China) 10 times with an inter-injection interval of 1 h and the last injection of caerulein was followed by immediate intraperitoneal injection of lipopolysaccharide (LPS; 5 mg/kg; #L2880, Sigma, USA) to build up severe acute pancreatis (SAP)[15]. In the groups of caffeine treatment, 25 mg/kg caffeine (#C0750, Sigma, China) was injected at the same time as caerulein injections (beginning from the fourth injections)[16]. Mice in the HTG group and HAP group were fed normal saline.

To confirm the central role of gut microbiota in caffeine protective effects against HAP, we applied FMT experiments on the basis of constructing germ-free mice. The specific operation process was shown in antibiotic treatment (ABX) and FMT methods. The two groups of mice (n = 6) were randomly divided: HAP + FMT-CON group and HAP + FMT-CON group. Mice in HAP + FMT-CON group were gavaged with feces from control mice and then induced HAP, while mice in HAP + FMT-CAF group were gavaged with feces from caffeine-treated mice and then induced HAP.

In the next series of core animal experiments, we explored the role of *F. prausnitzii* in HAP progession. A total of four groups of mice (n = 6) were randomly divided: HTG group (HTG), HTG with *F. prausnitzii* group (HTG + FP), HAP group (HAP) and HAP with *F. prausnitzii* group (HAP + FP). Mice received intraperitoneal injection of P-407 every 48 h for 4 weeks before experiment (from day 0 to 28) to induce HTG. The mice in HTG + FP and HAP + FP groups were oral gavaged with 5×10^8 CFU/mL of *F. prausnitzii* 1 week and HAP + FP was inducted to AP model[17]. The mice in the HTG group and the HAP group were fed normal saline 1 week before AP induction. On day 28, HAP and HAP + FP mice were intraperitoneally injected with caerulein combined with a single LPS injection to induce AP, while HTG and HTG + FP groups received equivalent saline injections as controls.

The mice induced AP were sacrificed 2 h after the injection of LPS. The rest of the mice were sacrificed at the same time. Pancreas, colons, cecum luminal contents and serum were collected immediately when being sacrificed.

2.5 ABX

To confirm the central role of gut microbiota in caffeine protective effects against HAP, we using ABX treatment to generate enteric germ-free mice. The mice were given antibiotic cocktail for 2 weeks. Antibiotic water bottles were inverted every day to keep the effectiveness of antibiotics. The mixture of antibiotic solution applied in this experiment consisted of following antibiotics: ampicillin (1 g/L, Sangon, China), neomycin (1 g/L, Sangon, China), metronidazole (1 g/L, Sangon, China), and vancomycin (0.5 g/L, Sangon, China).

2.6 FMT

In sterile conditions, stools of caffeine-treated mice and their control group were collected. A total of 100 mg stools from each group were resuspended in 1 mL of sterile saline under laminar flow hoods. A solution was mixed and shook for 10 s before centrifuging for 3 min at 800 × g. The supernatant was collected and transplanted into enteric germ-free mice by gastric irrigation for 2 weeks. To prevent bacterial composition from being altered, fresh transplant material was prepared on the same day of transplantation within 10 min before oral gavage.

2.7 Fecal DNA extraction and metagenomic analysis

For fecal DNA extraction and metagenomic analysis, total genomic DNA extraction from human fecal specimens of HTG and HAP patients was thoroughly conducted using the E.Z.N.A.® Soil DNA kit (Omega, GA, USA), strictly following the manufacturer's prescribed protocols. The integrity of the extracted DNA was verified through electrophoresis on a 1% agarose gel. Fragmentation to an average size of approximately 400 bp was achieved with Covaris M220, enabling the construction of a paired-end library utilizing the NEXTflex Rapid DNA-Seq kit (Bioo Scientific, Austin, TX, USA). Illumina NovaSeq sequencing at Majorbio Bio-Pharm Technology Co., Ltd. (China) facilitated the acquisition of high-quality paired-end reads.

Raw sequencing reads underwent meticulous preprocessing on the Majorbio Cloud Platform, involving adapter sequence removal, trimming, and elimination of low-quality reads utilizing fastp. Alignment against the human hg38 reference genome was conducted using BWA, effectively excluding reads of host origin. High-quality reads were assembled into contigs through MEGAHIT, leveraging succinct de Bruijn graphs. The final assembly considered only contigs exceeding 300 bp in length. Open reading frames (ORFs) within the contigs were identified employing MetaGene. For gene abundance evaluation, reads post-quality control were mapped to the non-redundant gene catalog with 95% identity through SOAP aligner.

Gene set annotation for bacteria was accomplished utilizing DIAMOND against the NCBI NR database. Each gene was allocated to the highest-scoring taxonomy, based on a unified database, facilitating a comprehensive assessment of microbial species within the gut ecosystem of individuals with recurrent abdominal pain. Selection of significantly different species was determined by VIP scores derived from the OPLS-DA model (VIP > 1). Additionally, linear discriminant analysis (LDA) effect size (LEfSe) with LDA score > 3.5 discerned distinctive bacterial species. To discern differences, the Kruskal-Wallis H test was meticulously applied.

The data can be found in the NCBI database with accession number: PRJNA1099407.

The association analysis of metagenomics and metabolites was conducted via Majorbio Cloud Platform. Information on intestinal bacteria and metabolites with significant differences and the highest relative abundance between HAP and HTG groups were collected. And then the spearman correlation analysis was carried out between them.

2.8 Histopathology

After the mice were sacrificed, their pancreas and colonic tissues were fixed in 4% formalin for 24 h prior to embedding in paraffin. Then the tissues were cut into 4 μm sections for staining with hematoxylin and eosin (H&E). Morphological alterations of pancreas and colons were estimated by different pathologists in a blinded manner with light microscope (Leica, Germany). The evaluation of pancreatic tissue alterations during AP adhered to scoring system established by the Schmidt criteria[18]. Details are shown in the supplementary information. Histological score of colons was calculated in accordance with established methodologies[19]. Globet cells were examined by staining periodic acid-Schiff in standard procedures.

2.9 Immunofluorescence

First, the antigen of the 4 μm sections was extracted by using boiling citrate buffer (pH 6.0) before inactivating the endogenous peroxidase by 3% H_2O_2 solution at room temperature for 15 min. Then sections were incubated with the primary antibody at 4 °C overnight: occludin (1:100, ab216327, Abcam, USA), claudin-1 (1:100, ab125028, Abcam, USA), lysozyme (1:100, A0099, Dako, Danmark), inducible nitric oxide synthase (iNOS; 1:100, ab178945, Abcam, USA) and arginase-1 (Arg-1; 1:100, 93668, CST, USA). Then, sections were incubated with fluorescein-labeled antibody (1:100, Yeason, Shanghai) for 30 min. At last, the nucleus was counterstained with DAPI for 10 min to display images.

2.10 Serum parameter assays

The activities of the serum amylase and lipase, and the concentrations of serum cytokines, interleukin (IL)-1β, tumor necrosis factor α (TNF-α), IL-6, were measured with commercial kits (Roche, Berlin, Germany) and Luminex Screening Assay (R&D Systems, MN, USA), respectively.

2.11 TUNEL staining

Apoptosis of colonic epithelial cells was measured with TUNEL assay kit (Roche, 11684817910, Mannheim, Germany). All cell counting was carried out at 200× magnification.

2.12 Western blotting

After homogenizing pancreas or colon tissues with lysis buffer (RIPA) and protease inhibitor cocktail, concentrations of proteins were detected by BCA protein quantitative assay (Beyotine Biotechnology, China). Following SDS-PAGE separation, the extracts were transferred to PVDF membranes, which were blocked with 5% skim milk for 1 h. Following overnight incubation with primary antibodies at 4 °C, secondary antibodies conjugated with HRP were added. The used primary antibodies in the experiment are listed as below: NLRP3 (15101, CST, USA), caspase-1 (24232, CST, USA), IL-1β (12242, CST, USA), TLR4 (A11226, Abclonal, China), p65 (A22331, Abclonal, China), myeloid differentiation primary response 88 (MyD88; A21905, Abclonal, China), *β*-actin (AC026, Abclonal, China).

2.13 Total RNA extraction and transcriptome sequencing

For transcriptome sequencing, the TRIzol® reagent (Invitrogen, USA) was used to extract total RNA from gut tissues of HAP mice treated with caffeine or not. Illumina HiSeq xten/NovaSeq 6000 sequencer (two 150 bp reads) was used to examine the quality of extracted RNA. RNA-seq library preparation was performed using Illumina's TruSeq™ RNA sample preparation kit (San Diego, CA, USA). Raw paired-end reads were trimmed and quality controlled using SeqPrep and Sickle. Afterward, TopHat was used to compare and analyze clean reads with the reference genome in orientation mode. Genomic DNA was cleaved with DNase I (TaKaRa, Japan). To identify differential expression genes (DEGs), fragments per kilobase of exons per million mapped reads (FRKM) method was used to calculate the transcript expression levels. By using KEGG Orthology Based Annotation System (KOBAS), KEGG pathways were analyzed. The data could be found in the NCBI database with accession number: PRJNA1098802.

2.14 Culture of F. prausnitzii

F. prausnitzii was cultured as previously described[17]. *F. prausnitzii* (ATCC 27766, Manassas, VA, USA) was anaerobic cultured in LYHBHI solid medium at 10% CO_2, 10% H_2, 80% N_2, and 37 °C for 24 h. Then bacteria were plated in LYHBHI liquid medium at specific serial dilutions and grown anaerobically for 24–48 h. A standard curve of bacterial CFU/mL and the absorbance (at 600 nm) of cultures at known concentrations were used to perform quantitative analysis of bacteria. *F. prausnitzii* was collected in anaerobic incubator at 4 ℃. After that, *F. prausnitzii* was washed with PBS buffer for 2 times. Adjusting the bacterial concentration to 5×10^8 CFU/mL and transferring to bottle with rubber stopper for backup.

2.15 Statistical analysis

All data were analyzed with GraphPad Prism 9.0 software (San Diego, CA, USA) and expressed as mean ± standard error of mean (SEM). Student's *t*-test was employed to compare the differences between two groups. One-way analysis of variance (ANOVA) was employed to compare the differences between more than two groups. Kruskal-Walli's test was used to analyze the data not conforming to the normal distribution. If $P < 0.05$, the statistical differences would be considered to be significant.

3. Results

3.1 Cohort characteristics

A cohort of 36 HTG patients and 35 HAP patients was recruited to explore the metabolic and microbial characteristics of HAP. The clinical and demographic features of two groups were estimated in Table S1. There was no significant difference between two groups in terms of serum TG levels and demographic indices such as gender, age and body mass index.

3.2 Metabolomics analysis and comparison between HAP and HTG patients

We first examined the metabolic characteristics of HTG and HAP groups and detected significant differences in the metabolite profiles of both groups (Fig. 1A). As shown in the PLS-DA score plots (Fig. 1B), samples were well clustered. The HTG and HAP groups showed great distinction in both positive and negative ion modes cation and anion mode. The differences of metabolites between the HTG and HAP groups were sequenced in heat map (Fig. 1C). Metabolomics analysis identified 241 significantly differently expressed metabolites (145 up and 96 down) between two groups (Fig. 1D).

KEGG enrichment and topology analysis showed that caffeine metabolism is extremely different between the HTG and HAP groups ($P < 0.001$) (Figs. 1E–F). As Fig. 1G shown, the level of metabolites related to caffeine metabolism also altered significantly between the HTG and HAP groups. Compared with HTG group, the level of caffeine and theophylline significantly decreased in HAP group ($P < 0.001$ or $P < 0.01$), while the abundance of paraxanthine was significantly increased ($P < 0.001$). The results indicated that the decrease in caffeine was most pronounced in the HAP group compared to the HTG group, which suggested caffeine had a potential protective role.

Previous research has demonstrated that caffeine can modulate the gut microbiota composition and improve intestinal barrier function[20-21]. To identify the association of caffeine with clinical parameters, we performed Spearman correlation analysis. We found that caffeine showed a moderate negative correlation with serum C-reactive protein (CRP, $r = -0.774$, $P < 0.05$, Fig. 2A), IL-6 ($r = -0.692$, $P < 0.05$, Fig. 2B), TNF-α ($r = -0.579$, $P < 0.05$, Fig. 2C), IL-8 ($r = -0.798$, $P < 0.05$, Fig. 2D) and diamine oxidase (DAO, $r = -0.790$, $P < 0.05$, Fig. 2F). Moreover, caffeine showed a strong negative correlation with serum lipase ($r = -0.860$, $P < 0.05$, Fig. 2E), *D*-lactate (*D*-LAC, $r = -0.834$, $P < 0.05$, Fig. 2G) and lactate dehydrogenase (LDH, $r = -0.820$, $P < 0.05$, Fig. 2H). According to the findings, alterations in caffeine are strongly associated with pancreatic and intestinal injury during HAP.

3.3 Caffeine alleviates pancreatic and intestinal injury in HAP mice

We established mice model to explore the effects of caffeine on HTG and HAP *in vivo* (Fig. 3A). As shown in Figs. 3B–C, injury of pancreas in the HAP group was significantly aggravated compared with that of the HTG group, as evidenced by increasing histopathological scores in pancreas ($P < 0.001$) and serum levels of amylase ($P < 0.001$) and proinflammatory cytokines (IL-1β, TNF-α, IL-6, and IL-17) ($P < 0.001$). Compared with the HAP group, histopathological pancreatic damage, levels of serum amylase levels and proinflammatory cytokines were alleviated in the HAP + CAF group ($P < 0.001$ or $P < 0.01$). Neither HTG nor HTG + CAF showed significant differences in these measures (Figs. 3B–C). The results implied that caffeine attenuated pancreatic damage and systemic inflammation caused by HAP.

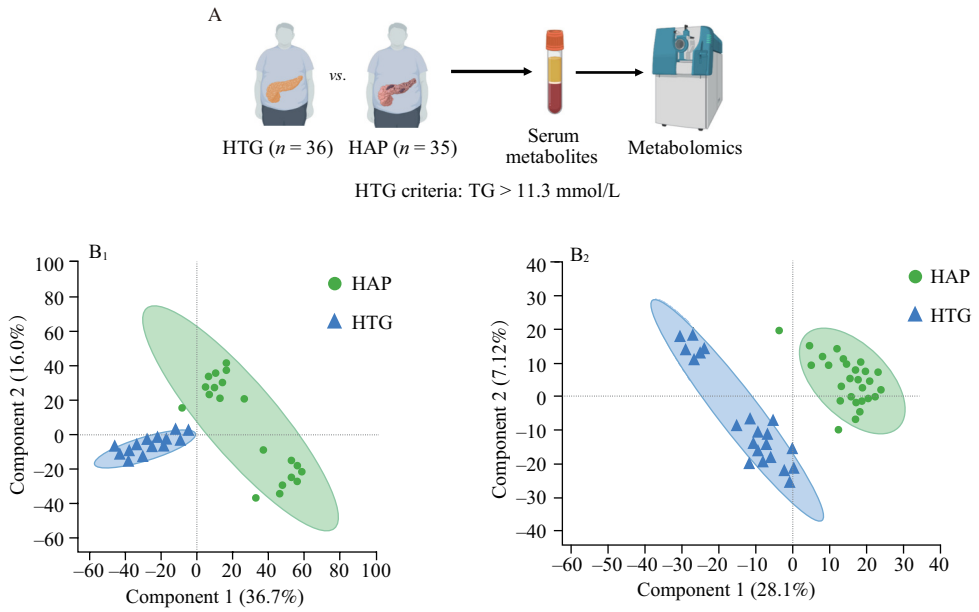
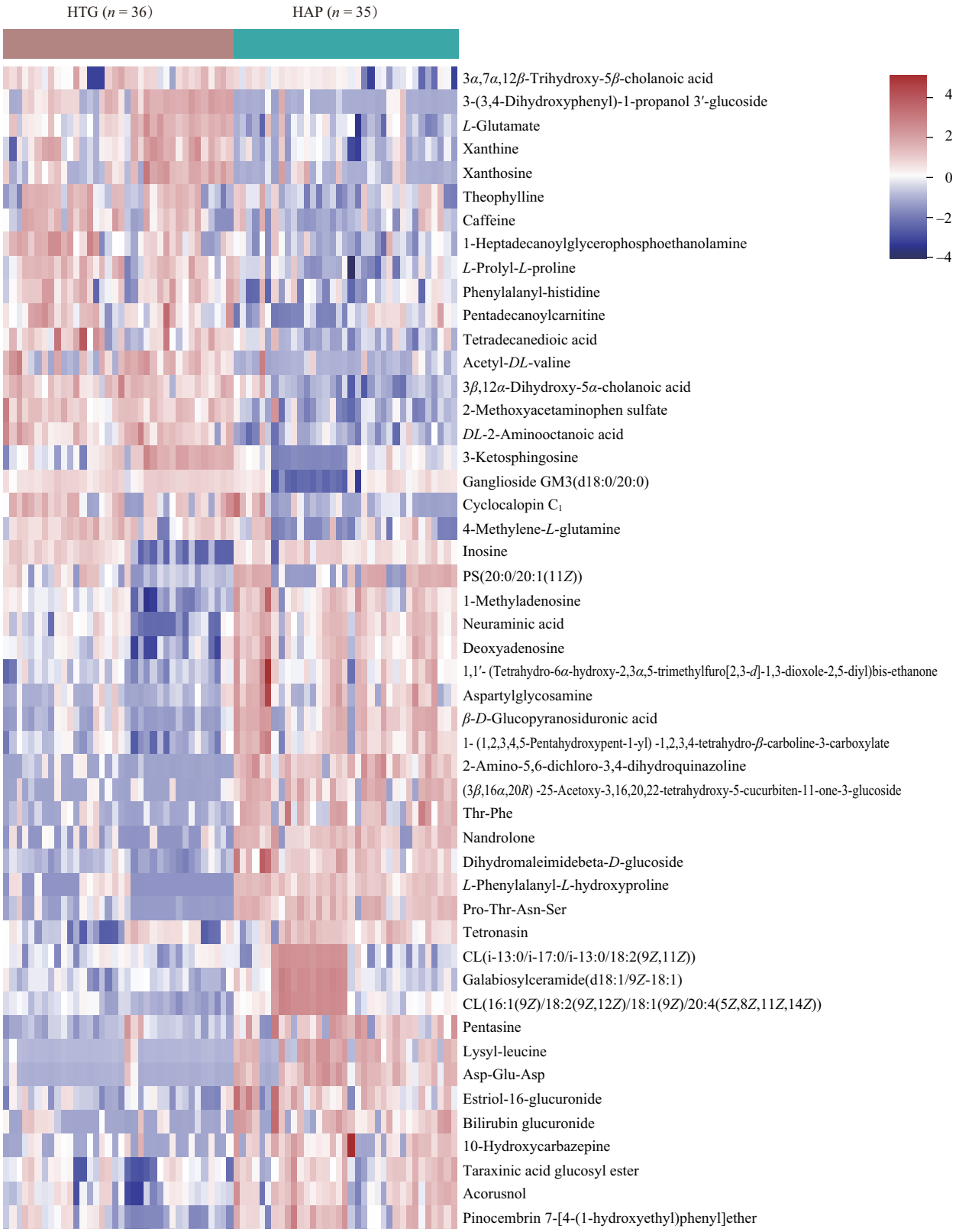


Fig. 1 Metabolic differences between HTG and HAP patients. (A) Metabolomics analysis of serum samples collected from the HTG group ($n = 36$) and the HAP group ($n = 35$). PLS-DA score plots among HTG and HAP groups in (B_1) positive and (B_2) negative ion modes. (C) Heat map showing differences of metabolites between HTG and HAP groups. (D) Volcano plot showing differences of metabolites between HTG and HAP groups. (E) KEGG enrichment analysis. (F) KEGG topology analysis. The abundance of (G_1) caffeine, (G_2) paraxanthine, and (G_3) theophylline in HTG and HAP groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

C



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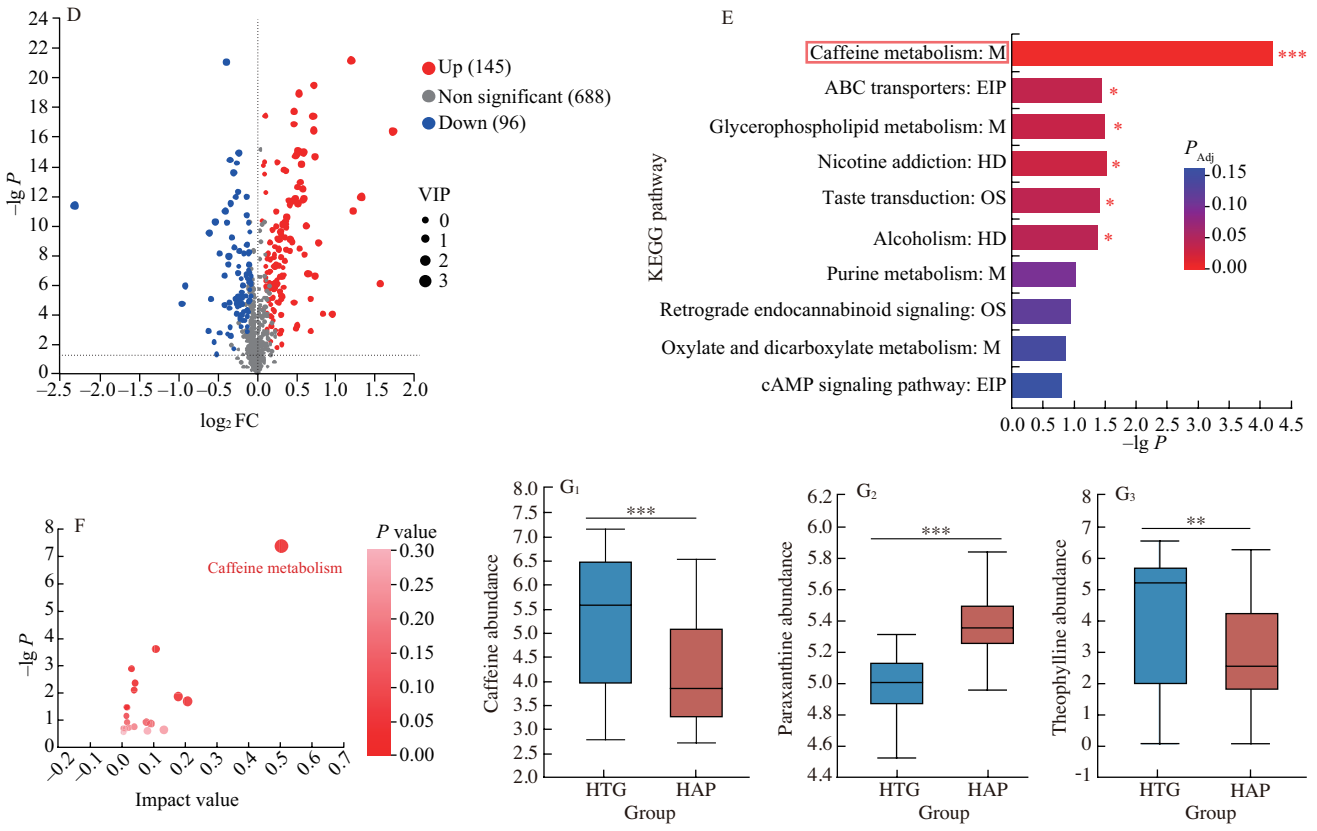

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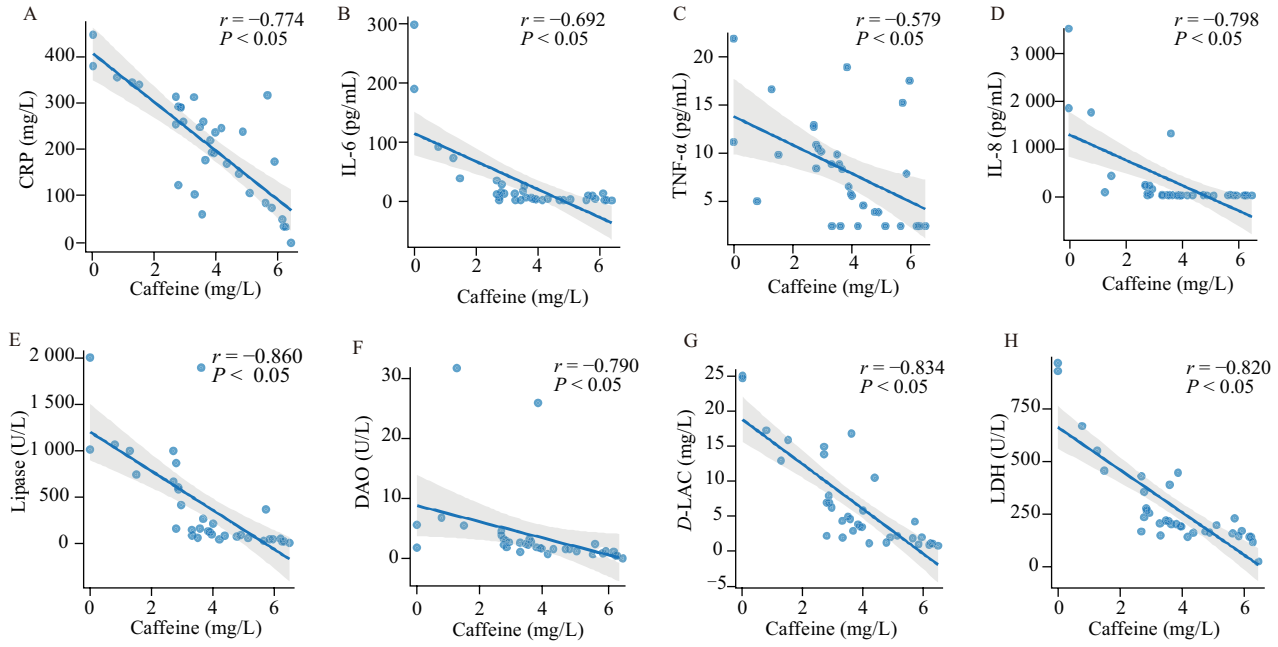


Fig. 2 Correlation analysis of caffeine with the clinical parameters of AP patients. Correlation analysis of caffeine with the inflammatory parameters, including (A) CRP, (B) IL-6, (C) TNF-α, (D) IL-8. (E) Correlation analysis of caffeine with lipase. Correlation analysis of caffeine with the parameters of intestinal permeability, including (F) DAO, (G) *D*-LAC, and (H) LDH.

We also investigated intestinal damage of HAP in our study. Gut injury was aggravated in the HAP group, as evidenced by severer histopathological injury of colons ($P < 0.001$) and ileums ($P < 0.001$), higher apoptosis of intestinal epithelial cells ($P < 0.001$) and the reduced expression of gut tight junction proteins like claudin-1 ($P < 0.01$) and occludin ($P < 0.01$) (Figs. 3D, S1A). Compared with the HAP group, the injury of gut was significantly attenuated in the HAP + CAF group. Paneth cells and goblet cells, as classical kinds of intestinal epithelial cells which significantly reduced in the HAP group ($P < 0.001$), were raised in HAP + CAF group ($P < 0.01$) (Fig. 3E). In the HAP group, the infiltration of type 1 macrophage (M1) which promotes inflammation increased ($P < 0.001$) while the infiltration of type 2 macrophage (M2) which inhibits inflammation decreased ($P < 0.001$). Caffeine reversed such alteration trends in the HAP + CAF group (Fig. S1B). The results indicated that caffeine could mitigate damage and macrophage infiltration in mice guts during HAP.

3.4 Caffeine alleviates HAP through a gut microbiota-dependent mechanism

Numerous reports have shown that caffeine has a significant effect on gut microbiota[22]. Considering the potential interaction of gut microbiota and caffeine, we hypothesize that attenuation of HAP by caffeine is mediated mainly through modulation of the gut microbiota, which subsequently leads to alleviation of pancreatic damage and intestinal homeostasis in the host. Therefore, we further explored the contribution of caffeine-regulated gut microbiota during HAP. Enteric germ-free mice produced by receiving ABX treatment were applied in our study. The stools from caffeine treated mice and its control group were collected and gavaged to ABX mice and then induced HAP (Fig. 4A). Compared with mice receiving feces from control mice, feces from caffeine-treated mice caused less severe pancreatic and ileal injuries in mice.

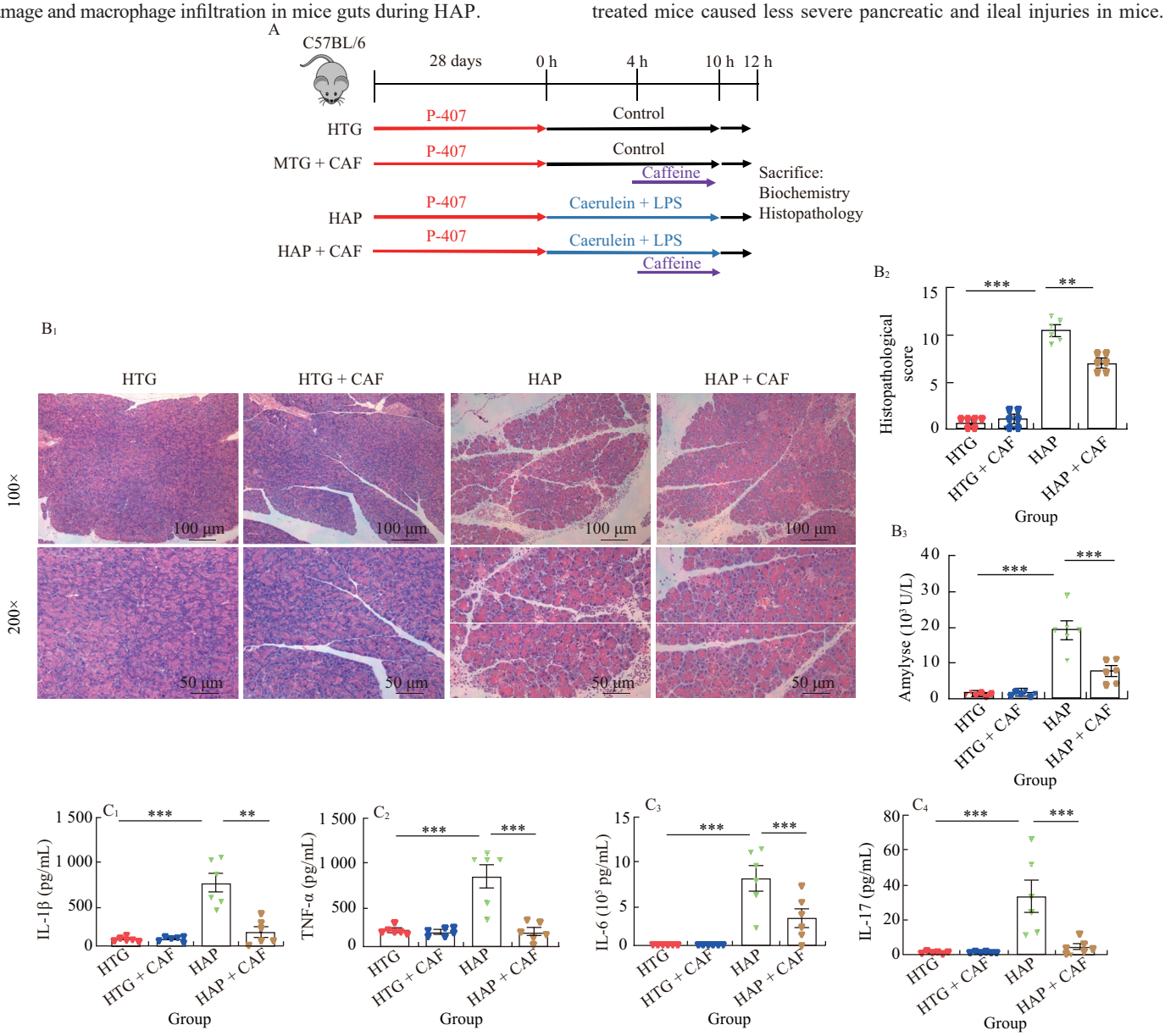


Fig. 3 Caffeine recovered the inflammation response and injury in pancreas and guts raised by HAP. (A) Schematic diagram of experimental design. (B) H&E staining and pathological scoring of pancreatic tissues in four groups. Serum amylase level was also displayed. Serum cytokine levels including (C_1) IL-1β, (C_2) TNF-α, (C_3) IL-6, (C_4) IL-17 in four groups. (D) Histological staining, immunofluorescence micrograph of claudin-1 and TUNEL micrograph of colonic tissues in four groups. (E) The number of Paneth cells and goblet cells were showed and calculated in four groups. Data are provided as the mean ± SEM ($n = 6$).**$P < 0.01$, ***$P < 0.001$.

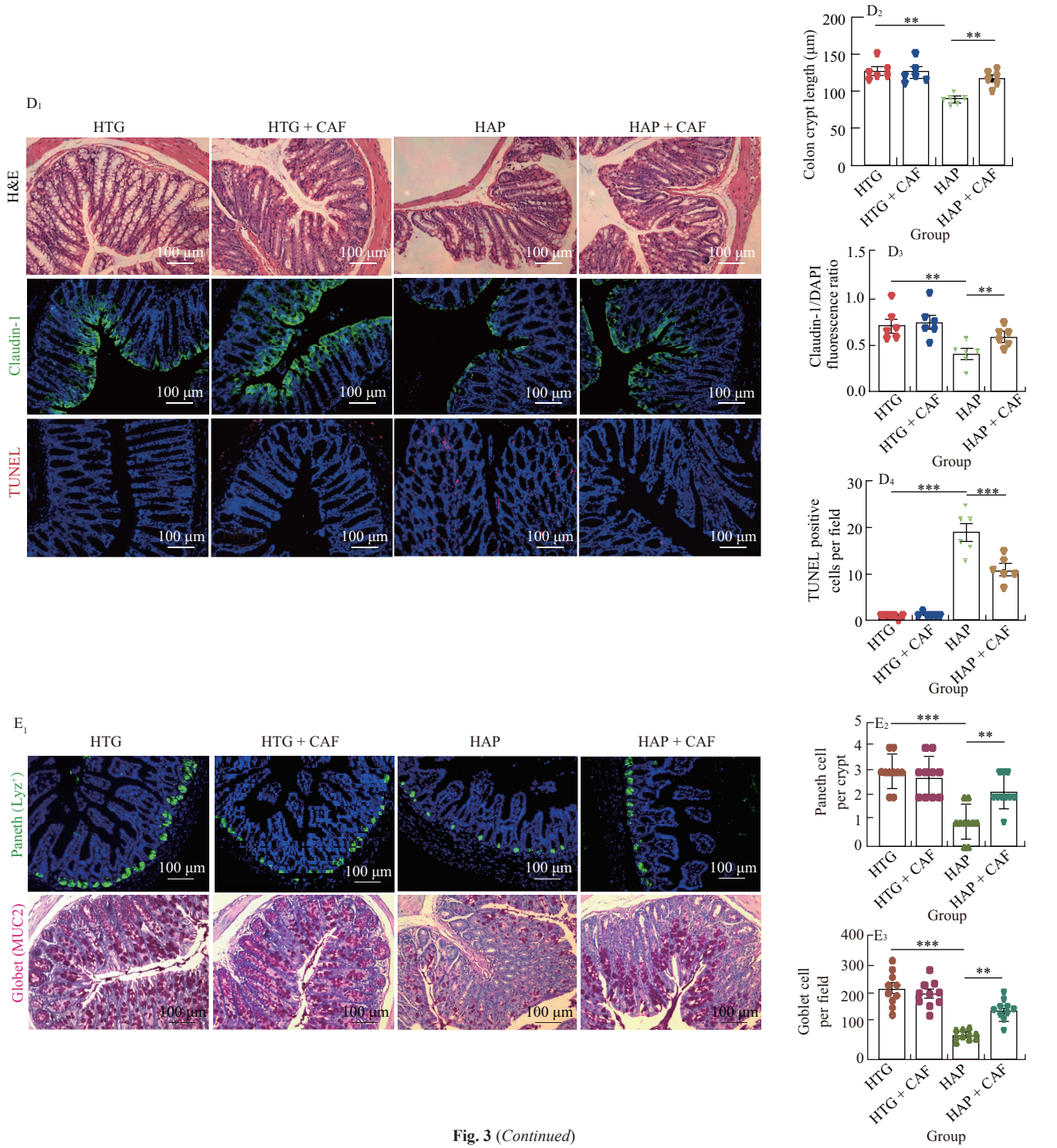


Fig. 3 (*Continued*)

Alleviation of pancreatic and gut injuries were evidenced by reduced pancreatic histopathological score, serum amylase level ($P < 0.001$) and recovered colon crypt length and intestinal barrier protein ($P < 0.01$) (Figs. 4A−B). According to the results, gut microbiota plays a key role in caffeine's ability to alleviate HAP.

To further verify this conclusion, we compared the effects of caffeine on HAP in normal and enteric germ-free mice and found that the protective effect of caffeine on HAP was significantly weakened in the absence of gut bacteria (Fig. S2). This finding reinforces the idea that gut microbiota may determine the efficacy of caffeine on HAP.

3.5 Gut microbiota composition differs in HAP and HTG patients

We proceeded to delve deeper into the microbial changes in HAP. Based on the Shannon index, the diversity of HAP experienced a significant reduction in comparison to HTG ($P < 0.05$, Fig. 5A). As principal coordinate analysis (PCoA) unveiled that the microbiota composition in HAP group was different from HTG group (Fig. 5B). Alterations in the microbial structure were revealed in HAP group at phylum and genus levels compared

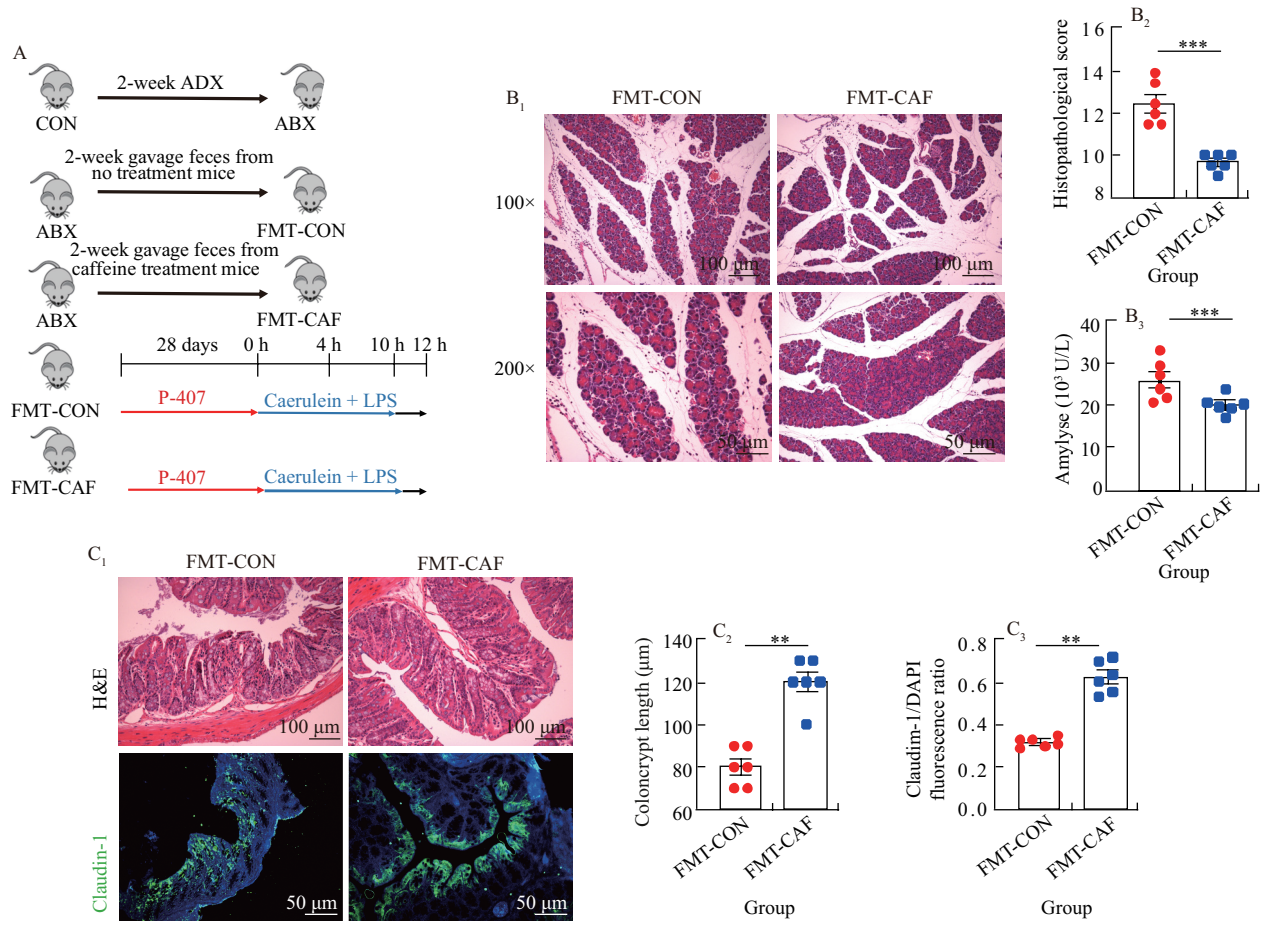


Fig. 4 Presence of gut microbiota is crucial for caffeine to alleviate HAP. (A) Schematic diagram of FMT experimental design. (B) H&E staining and pathological scoring of pancreatic tissues in FMT-CON and FMT-CAF groups. Serum amylase level was also displayed. (C) Histological staining and immunofluorescence micrograph of claudin-1 in colonic tissues for FMT-CON and FMT-CAF groups. Data are provided as the mean ± SEM ($n = 6$).**$P < 0.01$, ***$P < 0.001$.

with HTG group (Figs. 5C–D). Wilcoxon rank-sum test was further performed at genus-level to compare the gut microbiota discrepancy (Fig. 5E). Compared to the HTG group, the relative abundance of g_*Faecalibacterium* and g_unclassified_f_Lachnospiraceae were significantly decreased ($P < 0.05$), while the relative abundance of g_*Parabacteroides* and g_*Enterococcus* were significantly increased in the HAP group ($P < 0.05$) (Fig. 5F). Notably, the relative abundance of gut commensal *Faecalibacterium* ranking first in Wilcoxon rank-sum test showed significant reduction in the HAP group compared with HTG group ($P = 0.013$) (Figs. 5E–F, S3). We also conducted a Spearman correlation analysis to examine the relationship between the metabolic changes and the microbial changes (Fig. 5G). We surprisingly found that *Faecalibacterium* is closely associated with level of caffeine ($P < 0.01$).

3.6 *F. prausnitzii plays core role in the progression of HAP*

For a better understanding of the role *F. prausnitzii* plays in the alleviation of HAP by caffeine, we established the mouse model of *F. prausnitzii* pretreatment *in vivo* (Fig. 6A). Compared with the HAP group, the HAP + FP group showed that *F. prausnitzii* alleviated histopathological damage of pancreas and guts ($P < 0.01$) and reduced serum amylase level ($P < 0.001$) in HAP (Fig. 6B). Moreover, increased proinflammatory serum cytokines, such as IL-1β, TNF-α, IL-6 and IL-17 in the HAP group were notably decreased with *F. prausnitzii* treatment in the HAP + FP group ($P < 0.01$, Fig. 6C), suggesting its role in attenuating pancreatic damage. In addition to its protective effects on the pancreas, in the HAP + FP group, *F. prausnitzii* intervention reversed intestinal tissue damage and macrophage infiltration observed in HAP (Fig. 6D). The HAP + FP group exhibited a noticeable restoration of disrupted intestinal barriers, evidenced by increased expression of tight junction proteins like claudin-1 ($P < 0.05$, Fig. 6D) and occludin ($P < 0.01$, Fig. S4A). Additionally, the reduction of both Paneth cells and goblet cells in HAP group were ameliorated in the HAP + FP group ($P < 0.01$, Fig. 6E). Furthermore, in the HAP + FP group, *F. prausnitzii* alleviated macrophage infiltration in the gut of HAP mice, characterized by a reversal in the numbers of M1 and M2 macrophages ($P < 0.01$, Fig. S4B). Interestingly, the findings above from the *F. prausnitzii* intervention align with those of caffeine intervention.

To further verify the protective effect of *F. prausnitzii* and caffeine on HAP, we used another widely recognized HAP model, which applied *GPIHBP1* knockout mice to spontaneously develop HTG, on which an AP model was built. We found that caffeine (Figs. S5A–C) and *F. prausnitzii* (Figs. S5D–F) were effective in improving pancreatic and intestinal damage during HAP respectively.

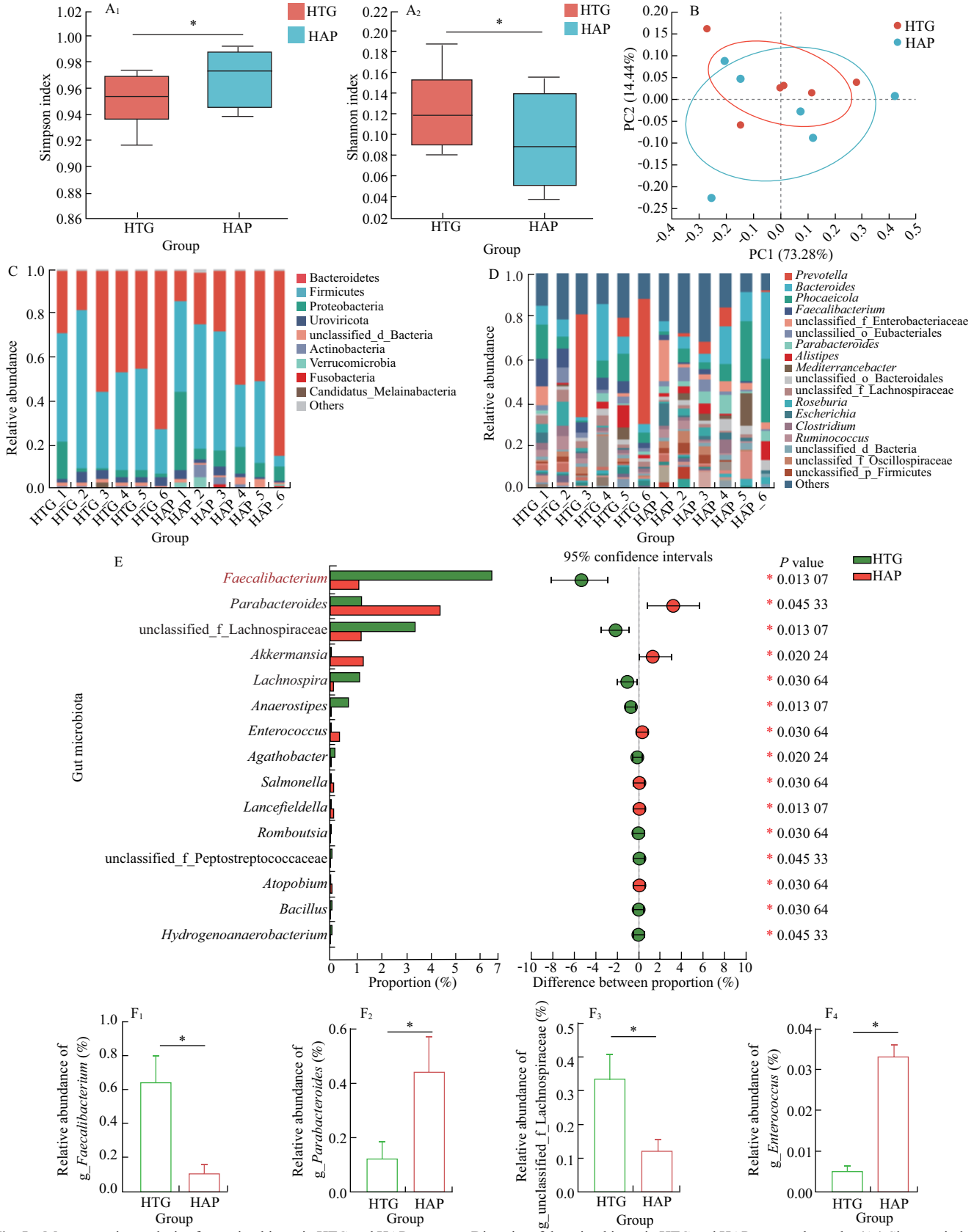


Fig. 5 Metagenomics analysis of gut microbiome in HTG and HAP groups. *α*-Diversity of the microbiome in HTG and HAP groups shown by (A_1) Simpson index and (A_2) Shannon index. (B) *β*-Diversity of the microbiome in HTG and HAP groups shown by PCoA score plot. (C) The microbial composition in the feces of HTG and HAP groups at phylum level. (D) The microbial composition in the feces of HTG and HAP groups at genus level. (E) The Wilcoxon rank-sum test for gut microbial changes between HTG and HAP groups at genus level. The differences of the abundance of (F_1) g_*Faecalibacterium*, (F_2) g_*Parabacteroides*, (F_3) g_unclassified_f_Lachnospiraceae, and (F_4) g_*Enterococcus* in the feces of HTG and HAP groups. (G) Spearman correlation analysis of fecal microbiome and serum metabolites. Data are provided as the mean ± SEM ($n = 6$). *$P < 0.05$, **$P < 0.01$.

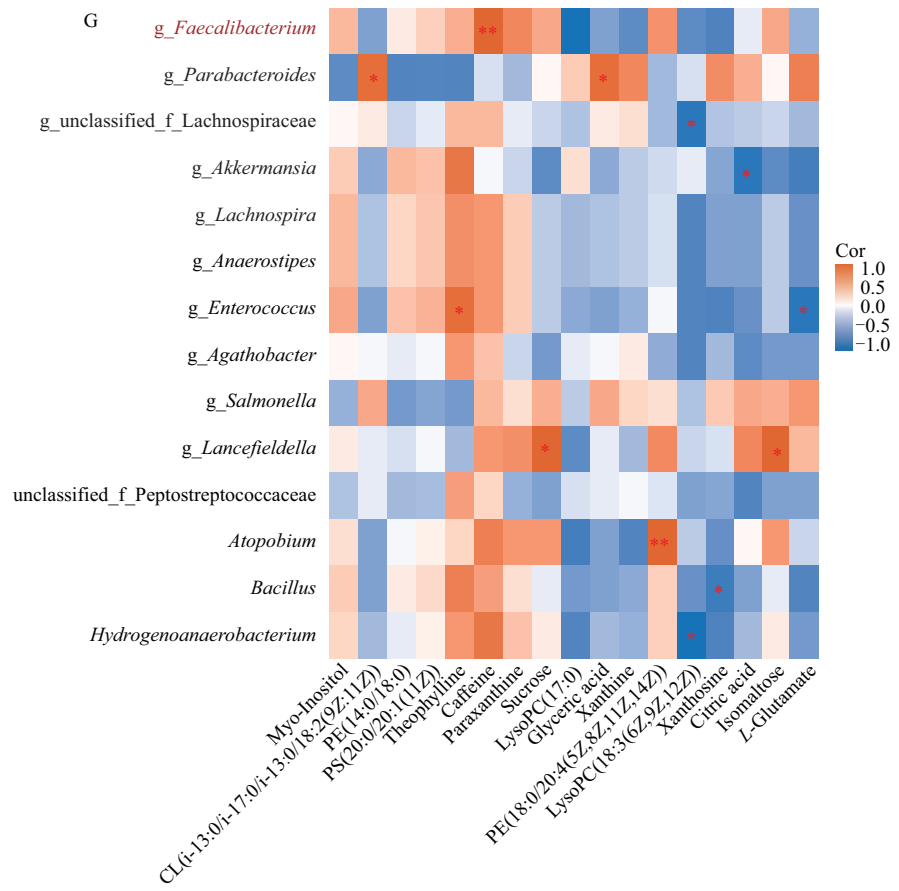


Fig. 5 (*Continued*)

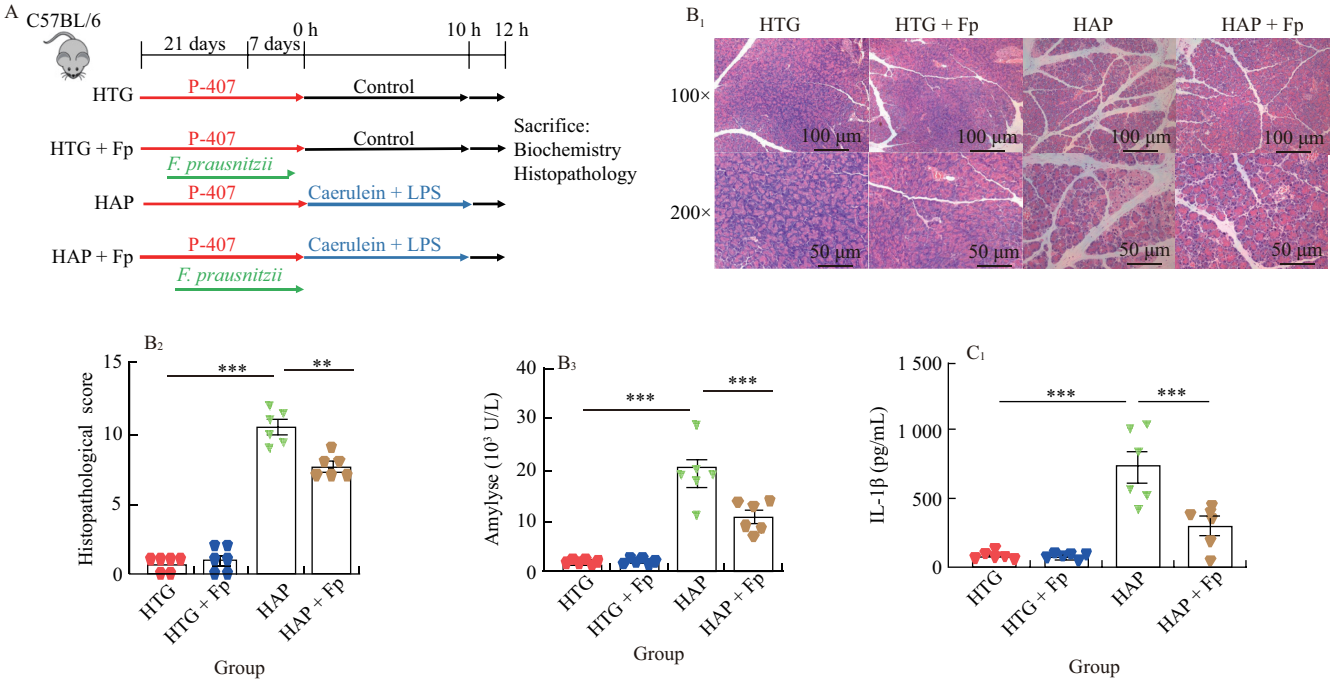


Fig. 6 *F. prausnitzii* recovered the inflammation response and injury in pancreas and guts raised by HAP. (A) Schematic diagram of experimental design. (B) H&E staining and pathological score of pancreatic tissues in four groups. Serum amylase level was also displayed. (C) Serum cytokine levels including IL-1β, TNF-α, IL-6, IL-17 in four groups. (D) Histological staining, immunofluorescence micrograph of claudin-1 and TUNEL micrograph of colonic tissues in four groups. (E) The number of Paneth cells and goblet cells were shown and calculated in four groups. Data are provided as the mean ± SEM ($n = 6$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

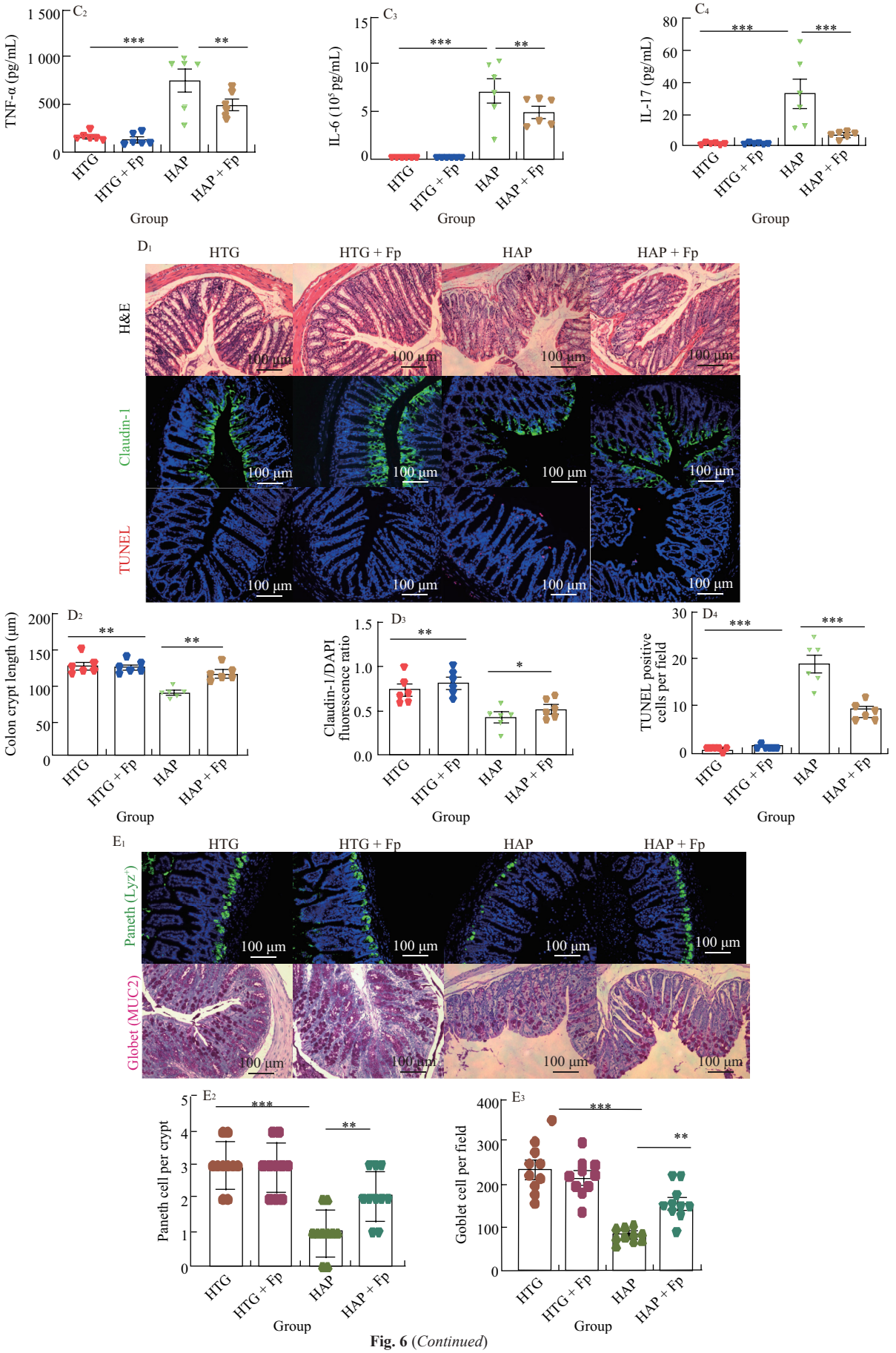


Fig. 6 (*Continued*)

3.7 Inhibition of NLRP3 inflammasome and TLR4-MyD88-mediated inflammatory response were required in protection against HAP by caffeine or F. prausnitzii

To investigate the mechanism underpinning the alleviation of HAP by caffeine and *F. prausnitzii* respectively, we conducted RNA sequencings to detect variations in gene expression of gut between the groups of HTG and HAP. There were 56 980 genes being detected, 1 313 were significantly regulated up, while 1 265 were significantly regulated down (Fig. 7A). The KEGG enrichment analysis identified that genes related to TLR, IL-17, NOD-like receptor and TNF signaling pathways were significantly altered between the HTG and the HAP groups (Fig. 7B). Similar analysis was conducted to detect the genetic alteration between the HAP and the HAP + CAF group (Fig. 7C). HAP and HAP + CAF groups differ significantly in their gene expression associated with NOD-like receptor, TLR, TNF and IL-17 signaling pathways (Fig. 7D). As TLR signaling pathway and NOD-like receptor signaling pathway are significantly enriched before and after caffeine intervention during HAP, indicating that genes related to these two pathways may play a critical role in the protective effects of caffeine against HAP. The deeper investigation of different expression genes in the HTG, HAP and HAP + CAF groups demonstrated the core genes in TLR signaling pathway and NOD-like receptor signaling pathway were associated with TLR4 and NLRP3 receptors (Fig. 7E).

TLR4/NLRP3 pathway was reported as intervention targets for some certain medicine treating AP[23]. In order to further clarify the role of *TLR4* in the alleviation of HAP by caffeine, we established the *TLR4* knockout mice. In comparison with the WT group, the *TLR4*$^{-/-}$ group showed that *TLR4* deficient mice of HAP showed less pancreatic and gut damage ($P < 0.01$ or $P < 0.001$, Fig. 7F). Western blot was performed to verify the pancreatic and intestinal expression of TLR4 and NLRP3 related proteins. Compared to the HTG group, the related proteins enhanced significantly in HAP group while the caffeine intervention or *F. prausnitzii* intervention mitigated such enhancement (Figs. 7G−H). Consequently, TLR4/NLRP3 may participate in the regulatory effects of caffeine or *F. prausnitzii* on HAP.

Taken together, our results indicate the metabolites caffeine have a central role in modulating the intestinal homeostasis and systemic inflammatory responses during HAP. Specifically, the protective effects of caffeine can be attributed to its modulation of gut microbiota, particularly *F. prausnitzii*, and its impact on the TLR4/NLRP3 signaling pathway (Fig. 8).

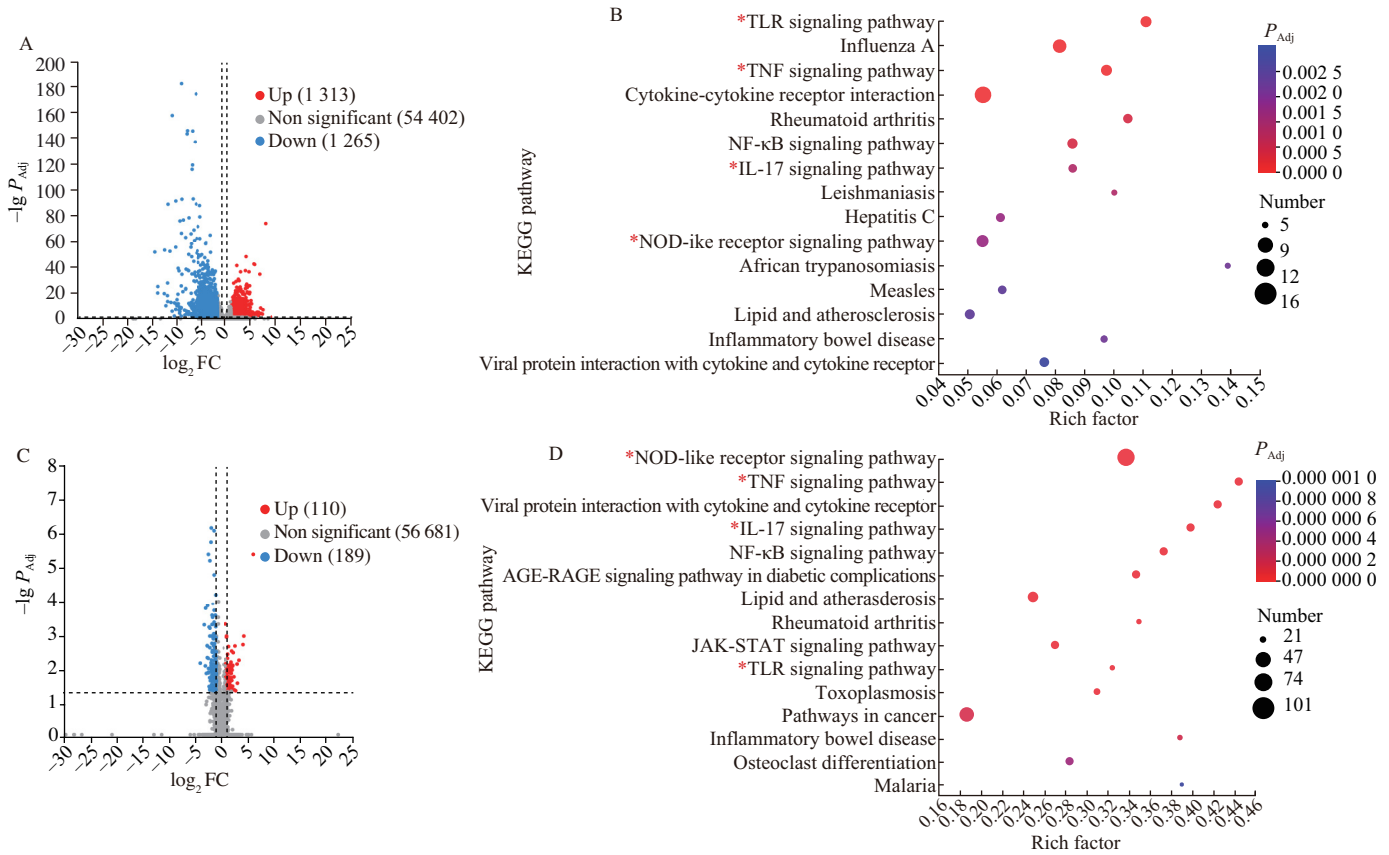
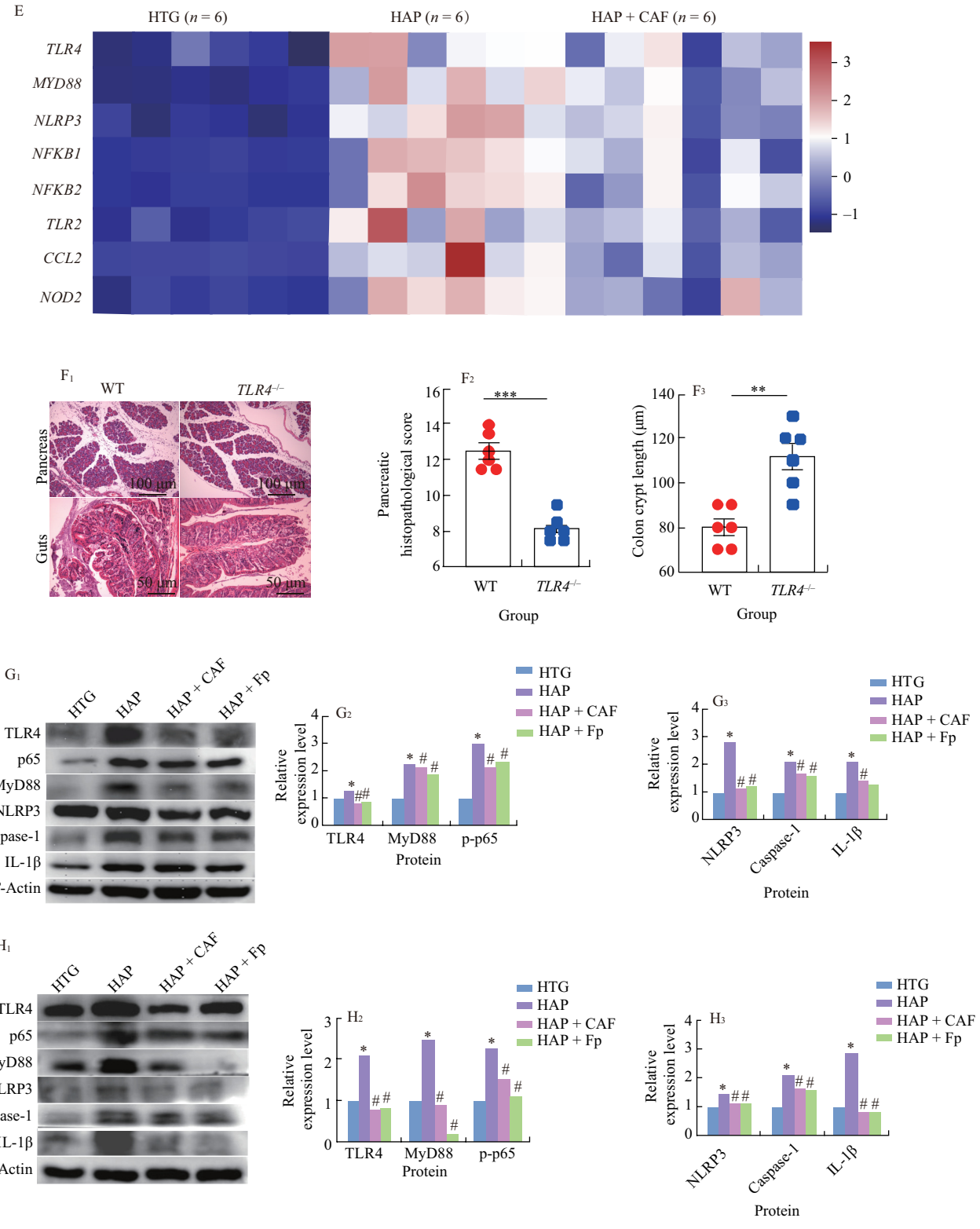


Fig. 7 Inhibition of NLRP3 inflammasome and TLR4-MyD88-mediated inflammatory response were required in protection against HAP by caffeine or *F. prausnitzii*. (A) Volcano plot showing significantly changed genes between HTG and HAP groups. (B) KEGG enrichment analysis between HTG and HAP groups. (C) Volcano plot showing significantly changed genes between HAP and HAP + CAF groups. (D) KEGG enrichment analysis between HAP and HAP + CAF groups. (E) Heat map showing gene differences between HTG, HAP and HAP + CAF groups. (F) H&E staining and pathological score of pancreatic and gut tissues in WT or *TLR4*$^{-/-}$ mice of HAP. (G) Western blot and its statistical chart showing the intestinal expressions of NLRP3, caspase-1, IL-1β, TLR4, p65, and MyD88. (H) Western blot and its statistical chart showing the pancreatic expressions of NLRP3, caspase-1, IL-1β, TLR4, p65, and MyD88. Data are provided as the mean ± SEM ($n = 3$ per group). $^{**}P < 0.01$, $^{***}P < 0.001$; $^{*}P < 0.05$, compared with HTG, $^{\#}P < 0.05$, compared with HAP.



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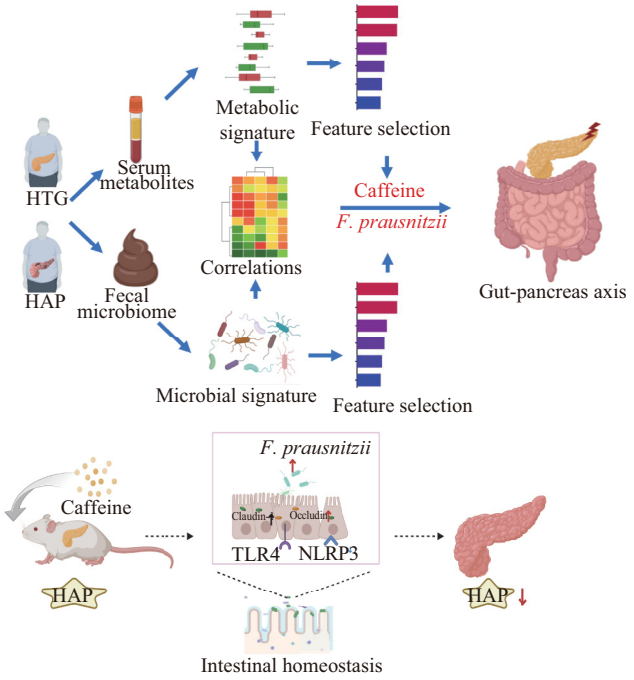

Fig. 8 In this study, we conduct metagenomics and metabolomics in HAP patients to reveals its metabolites and microbiota characteristics. *In vivo* experiments indicate that caffeine plays a central role in modulating the intestinal homeostasis and systemic inflammatory responses during HAP. Specifically, the protective effects of caffeine can be attributed to its modulation of gut microbiota, particularly *F. prausnitzii*, and its impact on the TLR4/NLRP3 signaling pathway.

4. Discussions

In our cohort study, caffeine levels and HAP severity were strongly correlated. Additionally, patients with HAP exhibited a reduced abundance of *F. prausnitzii*. In our murine models, caffeine demonstrated the ability to alleviate HAP and increase the abundance of *F. prausnitzii* through a gut microbiota-dependent mechanism. Besides, caffeine and *F. prausnitzii* protected the gut homeostasis to alleviate HAP via the suppression of TLR4/NLRP3 inflammasome pathway in gut and pancreas.

Increasing fat consumption alters gut microbiota and its metabolic products, resulting in non-alcoholic liver fatty disease and metabolic disorders[24-25]. HTG is a common and significant contributor of AP. HAP is the pancreatitis with TG more than 1 000 mg/L excluding biliary and alcoholic factors, which is easier to develop into SAP[26]. In several studies, a large role was played by gut microbiota in HAP[27]. While few studies of HAP are associated with its metabolic features. By using metabonomics sequencing, our study identified significant difference in human serum metabolites between HTG and HAP cohorts, especially the serum level of caffeine (Fig. 1). Importantly, it suggested that patients with hyperlipidemia who had lower levels of caffeine metabolites in their serum were more likely to suffer HAP.

Caffeine (1,3,7-trimethylxanthine), an alkaloid, which contains coffee and energy drink[28]. Although caffeine has been reported to mildly affect lipid metabolism by activating AMPK signaling and promoting lipolysis[29-30], these direct metabolic effects alone cannot fully explain its pronounced protection in HAP. Increasing evidence suggests that caffeine primarily exerts its beneficial effects through the modulation of the gut microbiota and the restoration of intestinal homeostasis[20-21]. Studies have found that caffeine regulates gut microbiota in obesity mice fed high-fat diets, thus improving metabolic syndrome[31]. Another study demonstrated that caffeine alleviated colitis via protecting the intestinal epithelial cell barrier[32]. As previously reported, caffeine can protect against AP by regulating Ca^{2+}[16]. However, few studies explored how caffeine attenuates AP from the perspective of intestinal homeostasis.

The study found that caffeine is associated with CRP, IL-6, TNF-α, IL-8, lipase, DAO, *D*-LAC, and LDH levels in HAP patients (Fig. 2). It implies that alterations of caffeine may be used to assess the severity of pancreatic and gut injury during AP in a hypertriglyceridemic state. Our results also confirmed that caffeine is effective in reducing HAP, improving intestinal barrier function, and affecting intestinal cells (Figs. 3 and S1). Therefore, we hypothesize that caffeine alleviates HAP by regulating intestinal homeostasis.

The effect of metabolites on diseases generally involves microbiota alteration in the gut. For example, the therapeutic effects of short chain fatty acid, a kind of critical metabolites that affect AP, are usually followed by an increase in beneficial gut microbes like *Bifidobacterium*[33]. It was found that caffeine increased the diversity and abundance of certain bacterial species in inflammatory bowel disease, including *Parabacteroides*, *Oscillibacter*, Lachnospiraceae, and Ruminococcaceae[34]. Based on these facts, it is a possible explanation that caffeine alleviates HAP through increasing certain gut microbes. The concept of commensal microbiota plays a prominent role in gut microbial studies. For instance, it has been demonstrated that the gut commensal *Bifidobacterium* exerts health-promoting effects by enhancing the integrity of the gut barrier and mitigating mucosal inflammation. Consequently, we analyzed metagenomic sequencing from both HAP and control fecal samples. Compared with controls, HAP patients had significantly less gut commensal *F. prausnitzii* (Fig. 5). Gut commensal *F. prausnitzii* had been paid a great deal

of attention in recent years. Several diseases have been linked to it, including inflammatory bowel disease, psoriasis, colorectal cancer, type 2 diabetes and others[35]. Given our finding that the presence of intestinal flora is crucial for caffeine in alleviating HAP in Figs. 4 and S2 and the closed association between caffeine and *Faecalibacterium* shown in Figs. 5 and S4, we hypothesized that *F. prausnitzii* may regulated by caffeine and play the core role in the progression of HAP. Our subsequent intervention experiment with *F. prausnitzii* substantiated this viewpoint (Fig. 6). We used another widely recognized HAP model, which applied *GPIHBP1* knockout mice to spontaneously develop HTG, on which an AP model was built. The data (Fig. S5) further verified the protective effect of *F. prausnitzii* and caffeine against HAP.

Previous studies demonstrated that inflammation signaling pathways are critical factors to regulate AP. TLR4 is important signaling pathway in AP. It is reported that high-fat diet aggravated AP via TLR4-mediated inflammation[36]. Besides, TLR4 pathway has been found to affect AP through protecting intestinal barrier[37]. NLRP3 is an important inflammasome in AP. NLRP3 could regulate the both systematic inflammatory response and anti-inflammatory compensatory mechanisms, leading to severer AP[38]. Numerous metabolites are found to affect AP through NLRP3 inflammasome such as butyrate, lactate, and free fatty acids[39-41]. TLR4/NLRP3 pathway was reported as intervention targets for some certain medicine treating AP[23]. By using transcriptome, we found TLR4/NLRP3 pathway was enriched and upregulated in HAP mice (Fig. 7). Experiments with *TLR4* knockout mice further confirmed the importance of TLR4 as a key receptor in HAP models. We found that the intervention of caffeine or *F. prausnitzii* inhibited TLR4 and NLRP3 pathway related protein expression both in guts and pancreas. Based on the above results, we hypothesized that caffeine alleviated HAP via regulating *F. prausnitzii* and suppressing TLR4/NLRP3 signaling pathways in pancreas and guts.

While conventional HAP therapies predominantly focus on TG reduction and symptomatic management[42], our findings unveil a paradigm-shifting mechanism through multi-omics-driven discovery: the gut microbial ecosystem acts as a gatekeeper of caffeine's therapeutic efficacy by orchestrating intestinal homeostasis. Crucially, we demonstrate that caffeine, a ubiquitous dietary compound, exerts its protection not through direct pharmacological action, but via microbial metabolic reprogramming that simultaneously suppresses pro-inflammatory TLR4/NLRP3 signaling (RNA-seq, $P < 0.001$). Our findings suggest that the protective effect of caffeine against HAP is primarily mediated through gut microbiota modulation, establishing *F. prausnitzii* as both a diagnostic biomarker and therapeutic effector. Our causal validation using germ-free models provides the first evidence that dietary components can functionally reconfigure gut microbial networks to mitigate organ-specific inflammation, offering a blueprint for developing microbiota-targeted nutritional interventions across metabolic-inflammatory diseases.

5. Conclusion

This study establishes caffeine as the first dietary modulator of the gut-pancreas axis with proven efficacy against HAP. Through integrated human-murine investigations, we decipher a hierarchical mechanism wherein caffeine-mediated enrichment of *F. prausnitzii* reprograms microbial metabolism to inhibit pancreatic NLRP3 inflammasome activation via TLR4 suppression. These findings transcend conventional HAP therapeutics by introducing microbial metabolic plasticity as a druggable target, with caffeine serving as a prototype for microbiome-reprogramming agents. The caffeine-microbiota-TLR4 axis not only redefines nutritional immunomodulation in pancreatitis but also provides a universal framework for precision nutrition strategies in metabolic disorders. By shifting therapeutic focus from symptom suppression to gut ecosystem engineering, our work opens new avenues for dietary-microbiome co-therapeutics in organ-specific inflammatory diseases.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Ethics approval and consent to participate

Our clinical study was ratified by the Chinese Clinical Trial Registry (2300069239). We conducted animal experiments according to the instructions of the Institutional Animal Care and Use Committee (IACUC) at Shanghai General Hospital (2020AW095).

Availability of data and materials

The raw data that support the findings of this study are openly available in the SRA database with reference number PRJNA1098802 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1098802/); PRJNA1099407 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1099407/).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://doi.org/10.26599/FSHW.2025.9250861.

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