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ORIGINAL RESEARCH

Combined Inhibition of Thrombosis by *Lactobacillus paracasei* and Clopidogrel

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Background: *Lactobacillus paracasei* (LP) may affect the efficacy of clopidogrel (CLP).

Methods: Forty Sprague-Dawley (SD) rats were randomly divided into control group, LP group, CLP group, LP (pretreatment) + CLP group, and CLP + LP(posttreatment) group (n=6-8). The administration doses of CLP and LP in rats were 6.75 mg/kg/d and 10^9 CFU/d, respectively, for 14 consecutive days. Tail vein blood was collected to detect blood drug concentration, platelet function. Then, a thrombosis model was constructed using 20% $FeCl_3$, the complete vascular occlusion time, thrombus weight, and thrombus inhibition rate, inflammatory factors, gut microbiota, short-chain fatty acids (SCFAs), trimethylamine N-oxide (TMAO) and mucosal barrier were evaluated.

Results: Compared with the CLP group, the blood concentrations of AM and CA in the combined group were significantly decreased, while platelet aggregation (MPA) and platelet reaction index (PRI) were significantly increased. After model construction, the thrombosis formation time was significantly prolonged, the thrombus weight was significantly reduced, and the thrombus inhibition rate was significantly; the secretions of TNF-α, IL-1β, P-selectin, GPIIb/IIIa, and D-dimer were significantly decreased in the combined group. The structure of gut microbiota also changed significantly after CLP treatment, and LP combined with CLP could improve the dysbiosis caused by CLP through increasing SCFAs and decreasing TMAO. In addition, the expressions of ZO-1, Occludin, and P-gp were increased in the combined groups. It should be noted that there is a directional discrepancy between the changes in platelet function indices (MPA and PRI) and in vivo thrombosis outcomes, which may be related to the multi-factorial regulation of in vivo thrombosis.

Conclusion: LP may regulate the structure of gut microbiota (increasing SCFA-producing bacteria and inhibiting TMAO-producing bacteria), thereby protecting the intestinal mucosal barrier, inhibiting inflammatory responses, and cooperatively acting with CLP to inhibit platelet activation and improve coagulation function, although the specific mechanism needs further verification.

Keywords: clopidogrel, *Lactobacillus paracasei*, gut microbiota, platelets

Introduction

Clopidogrel (CLP), a commonly used medication for coronary heart disease and post-percutaneous coronary intervention (PCI), plays a critical role in inhibiting platelet activation and aggregation. Widely applied globally, it has become one of the top-selling drugs worldwide.[1] However, CLP itself is biologically inactive and must be metabolized by multiple hepatic enzymes into a pharmacologically active 5-thiol product to exert its anti-platelet effects.[2] Extensive clinical practice and research have demonstrated significant interindividual variability in CLP efficacy. Some patients fail to achieve the expected anti-platelet effects at conventional doses, putting them at higher risk of cardiovascular events due to insufficient platelet inhibition; conversely, others may experience excessive platelet inhibition, leading to adverse reactions such as bleeding.

Traditional views attribute interindividual variability in drug efficacy primarily to factors like age, gender, genetics, and liver/kidney function.[3] For example, CYP2C19 gene polymorphisms significantly influence CLP metabolism. Patients carrying certain CYP2C19 gene variants exhibit reduced metabolic capacity for CLP, resulting in decreased levels of active metabolites and weakened anti-platelet effects.[4] However, even after accounting for these factors, a substantial proportion of interindividual efficacy differences remain unexplained.

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In recent years, with the advancement of microbiomics research, the gut microbiota—an essential component of the human microbial community—has increasingly become a research hotspot.[5,6] Growing evidence indicates that gut microbiota and their metabolites can affect drug absorption, metabolism, and excretion, altering the pharmacokinetics and pharmacodynamics of drugs in the body, and thus influencing drug efficacy and safety.[7] If CLP efficacy can be improved by modulating the gut microbiota, this could provide a more effective therapeutic approach for relevant patients, helping to enhance their quality of life and survival rates. Therefore, investigating the combined effects between gut microbiota metabolism and anti-platelet drugs is of significant theoretical and practical importance for addressing interindividual efficacy variability of anti-platelet drugs like CLP and achieving precision medicine in cardiovascular disease treatment.

Materials and Methods

Experimental Animals

A total of 40 adult healthy Sprague-Dawley (SD) rats (clean grade, weighing 250–300 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Before the experiment, the rats were fed with standard feed, individually housed in a controlled environment with normal humidity and temperature maintained at (25±1)°C, and provided free access to food and water. The study was approved by the Institutional Ethics Committee of Shandong Provincial Third Hospital (No: 2024–145SP2H) and was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Research Council, USA).

The 40 SD rats were randomly divided into five groups using a random number table method: control group, LP group, CLP group, LP + CLP group, and CLP + LP group, with 8 rats in each group. Medications were administered at fixed time intervals daily. Specifically, rats in the control group were given normal water and food; those in the LP group received oral gavage of LP (10^9 CFU/d); the CLP group received oral gavage of CLP (6.75 mg/kg/d); the LP + CLP group was first gavaged with LP for 5 days, followed by CLP; the CLP + LP group was first gavaged with CLP for 5 days, followed by LP. All groups were administered continuously for 14 days. During the experiment, 2 rats died of anesthesia accidents (2 from the CLP group), and 4 rats had fecal samples that failed microbial analysis due to improper collection. The clopidogrel bisulfate tablets used in the experiment were produced by Sanofi-Aventis (Hangzhou) Pharmaceutical Co., Ltd. LP (CGMCC no. 9800) was obtained from the China General Microbiological Culture Collection Center.

The primary endpoint of this study is thrombosis formation time. Referring to the standard deviation of thrombosis formation time (approximately 2.5 min) in previous similar studies,[1] setting $\alpha=0.05$, $\beta=0.2$, and the expected difference between the two groups is 3 min. Using the formula $n=2\times(Z\alpha/2 + Z\beta)^2\times\sigma^2/\delta^2$, the required sample size per group is calculated to be 6. Therefore, the actual effective sample sizes of n=6 (for some experiments) and n=4 (for microbiome analysis) can meet the statistical power requirements for the primary endpoint.

Liquid Chromatography-Mass Spectrometry (LC-MS) for CA and AM Blood Concentration Detection

Four hours after the last administration, tail vein blood was collected from the rats and placed into EDTA anticoagulation tubes. Acetonitrile solution was immediately added, and the mixture was left standing at room temperature for 30 min, then centrifuged at 1000×g for 10 min to discard the precipitate. The concentrations of CA and AM in plasma were determined using an Agilent 1100 liquid chromatography system (Agilent, USA) and an API4000 tandem mass spectrometer, with data analyzed by Analyst 1.3 software (GE, USA).

For CA detection, 100 μL of clopidogrel carboxylic acid (CA) standard (National Institutes for Food and Drug Control, China; purity: 99.8%) or 100 μL of plasma sample was mixed at room temperature, followed by adding 100 μL of deuterated clopidogrel carboxylic acid standard solution (National Institutes for Food and Drug Control, China; purity 99.8%, 50 ng/mL) and 200 μL of methanol solution. After mixing, the sample was centrifuged at 4000 r/min (centrifugal radius 19 cm) for 10 min. Then, 100 μL of the supernatant was taken, mixed with 400 μL of pure water, and transferred to a vial for injection.

For AM detection, CLP active sulfhydryl metabolite derivative (AM) biological reference substance (Toronto Research Chemicals, Canada) or 0.3 mL of plasma sample was mixed at room temperature, followed by adding 100 μL of ticlopidine methanol solution (Sigma-Aldrich, USA; purity 99.4%, 0.6 mg/mL) and 400 μL of formic acid solution, pending detection.

Detection of MPA and PRI

At the end of the last administration, tail vein blood was collected from the rats. One portion of the blood was placed into sodium citrate vacuum collection tubes for determining maximal platelet aggregation (MPA) using a Model 700 platelet aggregometer (Chrono-Log, USA) and adenosine diphosphate (ADP, final concentration 5 μmol/L) was used as the agonist.

Another portion of the blood was placed into anticoagulation tubes containing EDTA and EDTA-4% formaldehyde, respectively, for determining the platelet reaction index (PRI). The red blood cells and platelets of the rats were counted using a Sysmex XE-200 hematology analyzer (Sysmex, Japan). PRI was calculated using the formula: PRI = [platelet count (EDTA)/platelet count (EDTA-4% formaldehyde)]×K, where K is the correction coefficient, K = [red blood cell count (EDTA)/red blood cell count (EDTA-4% formaldehyde)].

MPA and PRI were detected 4 hours after the last administration on day 14, and the $FeCl_3$-induced carotid artery thrombosis model was constructed immediately after blood collection for MPA and PRI detection. This time point was chosen because it is consistent with the peak concentration time of clopidogrel in rats (previous studies have shown that the peak blood concentration of clopidogrel in rats occurs 3–5 hours after oral administration), ensuring that the detection results can reflect the effect of the drugs at their effective concentrations.

Construction of $FeCl_3$-Induced Carotid Artery Thrombosis Model and Related Detection

After the treatment period, the rats were anesthetized, and the left common carotid artery was isolated. A 20% $FeCl_3$ filter paper was applied locally for 5 minutes, and the time to complete vascular occlusion was recorded. Twenty-four hours after surgery, thrombi were harvested and weighed to evaluate the effects of different treatment groups on thrombosis.

Gut Microbiota Diversity Analysis

Fresh fecal samples were collected after constructing the thrombosis model and stored at −80°C. DNA was extracted using the QIAamp DNA Stool Mini Kit, and the V3-V4 region was amplified for sequencing on an Illumina NovaSeq platform. OTU clustering was performed using QIIME2 to calculate α-diversity (Shannon index) and β-diversity (PCA analysis), and LEfSe analysis was used to identify differentially abundant microbiota, thereby exploring changes in the structure and diversity of gut microbiota among different treatment groups.

For beta diversity analysis of gut microbiota, permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance was performed to test the differences in microbial community structure among groups. For LEfSe analysis, the q-value (false discovery rate-adjusted p-value) was calculated to identify differentially abundant microbiota, with $q < 0.05$ considered statistically significant.

Gas Chromatography-Mass Spectrometry (GC-MS) for Short-Chain Fatty Acids (SCFAs) and TMAO Detection

For serum sample collection, after rats were anesthetized, tail vein blood was collected from the rats and placed into EDTA anticoagulation tubes. The blood was allowed to stand at room temperature for 30 minutes, then centrifuged at 3000×g for 15 minutes at 4°C. The upper layer of clear liquid was aspirated as serum and stored at −80°C until detection. For serum or fecal samples, 200 μL of serum or 0.1 g of feces was mixed with an internal standard (d4-butyric acid), extracted with diethyl ether, and concentrated by nitrogen blowing. The analysis was performed using an Agilent DB-FFAP column (30m×0.25mm×0.25μm). The inlet temperature was set at 250°C, the split ratio was 10:1, and the injection

volume was 1 μL. Helium was used as the carrier gas in constant flow mode at 1.2 mL/min. The temperature program was as follows: initial temperature 50°C held for 2 min, then increased to 140°C at 10°C/min, followed by an increase to 240°C at 5°C/min and held for 5 min. Electron ionization (EI) was performed at 70 eV, with the ion source temperature set at 230°C and the quadrupole temperature at 150°C. Selected ion monitoring (SIM) was used for SCFAs (acetic acid, propionic acid, butyric acid) and TMAO at specific m/z values to analyze the level changes of these metabolites in different treatment groups.

Intestinal Mucosal Permeability Detection

After constructing the thrombosis model, rats were sacrificed by cervical dislocation, and ileal tissues were collected, embedded in OCT, and prepared into 5-μm frozen sections. The sections were incubated with primary antibodies against ZO-1 (1:200, ab307799, abcam) and Occludin (1:150, ab216327, abcam) at 4°C overnight, followed by incubation with FITC-conjugated secondary antibodies (1:500, ab6717, abcam) in the dark. Fluorescence intensity and distribution were observed using a confocal microscope and analyzed by ImageJ to evaluate changes in intestinal mucosal permeability.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA kits for TNF-α, IL-1β, and IL-10 (Elabscience, Wuhan, China), P-selectin (Jianglai Bio, Shanghai, China), platelet membrane glycoprotein GPIIb/IIIa (Jingkang, Shanghai, China), and D-dimer (Sangon Biotech, Shanghai, China) were used according to the manufacturer's instructions for detection.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using the Trizol method (Takara, Shanghai, China), and reverse transcription was performed using the PrimeScript™ RT reagent Kit (Takara, Shanghai, China). PCR amplification was carried out using 2×SYBR Green qPCR Master Mix (Bimake, Texas, USA). The $2^{-\Delta\Delta CT}$ method was used to calculate the differential mRNA transcription levels of target genes. Primer sequence GAPDH (F: 5′- CATCACTGCCACCCAGAAGACTG −3′ and R: 5′- ATGCCAGTGAG CTTCCCGTTCAG −3′), P-gp (F: 5′-GCTTCTACAACCCCATGGCT −3′ and R: 5′- CGACAGCTGAGTCCCTTTGT-3′); ZO-1 (F: 5′-ACCTTGTTGGAGCCAACTGT −3′ and R: 5′- TCAGGGGCATGCTCACTAAC −3′) and Occludin (F: 5′-CAACGGCAAAGTGAATGGCA −3′ and R: 5′-CTTTCCCCTTCGTGGGAGTC were synthesized by Shanghai Sangon Bioengineering Co., Ltd.-3′).

Statistical Methods

Statistical analysis was performed using SPSS 19.0 software and R 4.3.0 software. First, normality of measurement data was tested using the Shapiro–Wilk test, and homogeneity of variance was tested using Levene's test. Data conforming to normal distribution and homogeneous variance were expressed as mean ± standard deviation (x±s). One-way analysis of variance (ANOVA) was used for comparisons among multiple groups, followed by Tukey's post-hoc test for pairwise comparisons, and Bonferroni correction was applied for multiple comparisons to control the type I error rate. Independent samples *t*-tests were used for comparisons between two groups. For beta diversity analysis of gut microbiota, permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance was performed to test the differences in microbial community structure among groups. For linear discriminant analysis effect size (LEfSe) analysis, the q-value (false discovery rate-adjusted p-value) was calculated to identify differentially abundant microbiota, with $q < 0.05$ considered statistically significant. A *p*-value < 0.05 was considered statistically significant for other analyses.

Results

Effects of LP and CLP on MPA and PRI in Rats

After 14 days of continuous feeding, there was no significant difference in body weight changes among groups (Figure 1A). Four hours after drug treatment on day 14, tail vein blood was collected to detect drug concentrations. The linear range for CA detection was 50.00–2500.00 ng/mL (r=0.9913), and for AM detection was 0.05–10.00 ng/mL

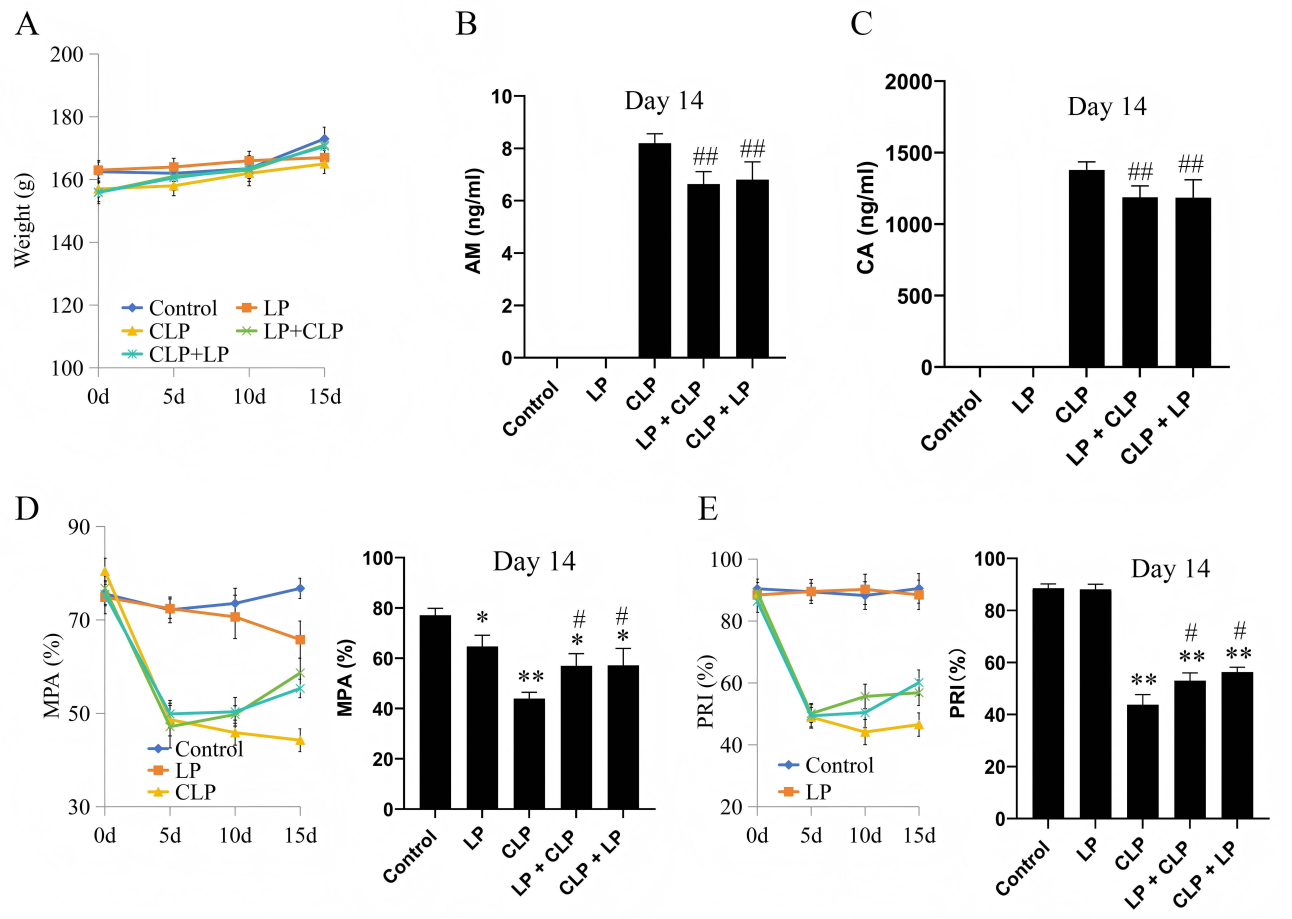

Figure 1 Effects of combined LP and CLP on MPA and PRI (n=6). (**A**) Body weight changes over different treatment periods; (**B** and **C**) AM and CA concentration Changes in blood on day 14; (**D**) MPA detection over different treatment periods; (**E**) PRI detection over different treatment periods. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test with Bonferroni correction. *$p < 0.05$ and **$p < 0.01$ vs Control; # $p < 0.05$ and ## $p < 0.01$ vs CLP.

(r=0.9989). Compared with the CLP group, the blood concentrations of AM in both the LP + CLP and CLP + LP groups were significantly lower ($p < 0.01$), and CA blood concentrations also decreased significantly ($p < 0.01$). However, the treatment order of LP and CLP had little effect on CLP metabolism (Figure 1B and C).

For MPA detection, significant differences among groups became apparent after 14 days of treatment. Compared with the control group, the LP single-treatment group showed a decrease in MPA, but no significant difference in PRI. The MPA and PRI in the CLP group, LP + CLP group, and CLP + LP group were significantly lower than those in the control group ($p < 0.05$). However, compared with the CLP group, the MPA and PRI in both the LP + CLP and CLP + LP groups were significantly higher ($p < 0.05$), with no statistically significant difference between the two combination groups (Figure 1D and E). These results suggest that single use of LP can inhibit MPA without obvious effects on PRI activation, while the combination of LP and CLP increases MPA and PRI in CLP-treated rats, regardless of the treatment order.

Effects of LP and CLP on Thrombosis in Rats

Using the $FeCl_3$-induced carotid artery thrombosis model, after model construction, there was no significant difference in thrombosis formation time in the LP group. The thrombosis formation time was significantly prolonged in the CLP group, LP + CLP group, and CLP + LP group. The carotid thrombosis formation time in the LP + CLP group (32.13 ±1.21 min) and CLP + LP group (30.55±1.73 min) was significantly longer than that in the CLP group (28.40±0.57 min) ($p < 0.01$) (Figure 2A). Compared with the control group, thrombus weight was significantly reduced in all other groups, indicating that LP alone had a protective effect. Compared with CLP alone, the combination therapy effectively inhibited

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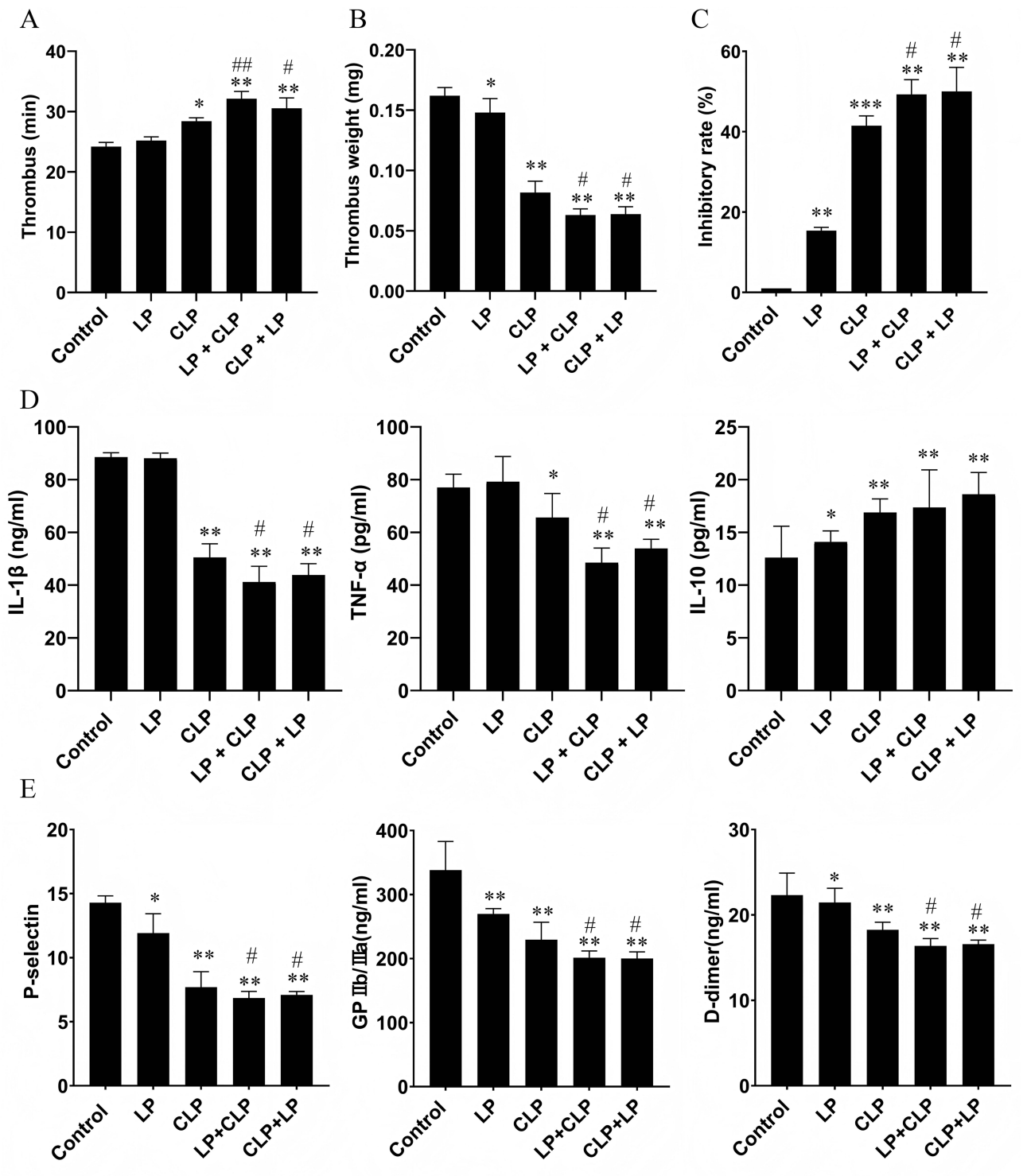

Figure 2 Inhibitory effects of combined LP and CLP on thrombosis in rats after model establishment (n=6). (**A**) Thrombosis formation time; (**B**) Thrombus weight; (**C**) Thrombus inhibition rate; (**D**) Serum concentrations of TNF-α, IL-1β, and IL-10 detected by ELISA; (**E**) Secretion of P-selectin, GPIIb/IIIa, and D-dimer detected by ELISA. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test with Bonferroni correction. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs Control; # $p < 0.05$ and ## $p < 0.01$ vs CLP.

thrombosis, with no effect of administration order on efficacy (Figure 2B).Two-way ANOVA analysis showed a significant interaction between CLP and LP on thrombosis formation time (F=5.28, p=0.028) and thrombus weight (F=4.96, p=0.035), indicating that the combined effect of CLP and LP on these endpoints is not simply the sum of their

individual effects. The effect size (partial η^2) of the interaction was 0.21 for thrombosis formation time and 0.19 for thrombus weight, suggesting a moderate interaction effect.

Compared with the control group, the thrombus inhibition rate was significantly increased in all other groups. The inhibition rate of LP alone was (15.38±0.81)%, that of CLP was (41.5±2.45)%, that of LP + CLP was (49.25±3.68)%, and that of CLP + LP was (50.00±5.99)%. The combination therapy showed a higher inhibition rate than CLP alone (Figure 2C).

After model construction, compared with the control group, single LP treatment caused no significant changes in serum TNF-α and IL-1β concentrations but significantly increased IL-10 concentration. Compared with the CLP group, the LP + CLP and CLP + LP groups showed further decreases in TNF-α and IL-1β secretion, with no significant difference in IL-10 (Figure 2D). Two-way ANOVA analysis showed a significant interaction between CLP and LP on serum TNF-α concentration ($F=4.82$, $p=0.038$), with an effect size (partial η^2) of 0.18. Platelet activation indices showed that LP alone inhibited the secretion of P-selectin, GPIIb/IIIa, and D-dimer compared with the control group. Compared with the CLP group, the combination of LP and CLP further reduced the secretion of these markers, indicating that the combination effectively inhibited platelet activation (Figure 2E).

Changes in Gut Microbiota Composition

Next, 16S rRNA sequencing analysis was performed on fecal samples after model establishment to determine the bacterial composition and structure in different treatment groups. Alpha and beta diversity results showed that gut microbial diversity in the LP group was higher than that in the control group, while CLP treatment significantly decreased gut microbiota diversity. The combination of LP and CLP increased alpha and beta diversity in the CLP-treated group (Figure 3A and B). PCA plots showed clear differences among the control, LP, and CLP groups, while the combination treatment groups trended toward the LP group, indicating that LP can reverse CLP-induced gut microbiota dysbiosis (Figure 3C). Figure 3D and E show the microbial community structure and composition at the family and genus levels.

At the family level, compared with the normal control group, the CLP group showed an increase in Lachnospiracea from 2.7% to 18.0% and Ruminococcaceae from 12.9% to 24.0%, which decreased after LP treatment. Muribaculaceae decreased from 16.8% to 9.9% in the CLP group and increased after LP intervention (Figure 3D). At the genus level, Turicibacter in the CLP group decreased from 9.1% to 2.1% and increased after LP intervention, with other genera showing consistent changes with their corresponding families (Figure 3E).

RT-qPCR detection of LP colonization in the intestine showed effective colonization in the LP, LP + CLP, and CLP + LP groups, while LP levels decreased in the CLP group (Figure 3F). Further analysis revealed that serum SCFAs were significantly increased ($p < 0.01$) and TMAO concentrations were significantly decreased ($p < 0.05$) in the LP, LP + CLP, and CLP + LP groups, indicating that the combination of LP and CLP significantly altered gut microbiota structure (Figure 3G and H). Two-way ANOVA analysis showed a significant interaction between CLP and LP on serum SCFAs concentration ($F=5.11$, $p=0.031$), with an effect size (partial η^2) of 0.20. Correlation analysis showed that serum SCFAs concentration was positively correlated with thrombosis formation time ($r=0.68$, $p<0.01$) and negatively correlated with thrombus weight ($r=-0.63$, $p<0.01$) and serum TNF-α concentration ($r=-0.59$, $p<0.01$). In contrast, serum TMAO concentration was negatively correlated with thrombosis formation time ($r=-0.61$, $p<0.01$) and positively correlated with thrombus weight ($r=0.57$, $p<0.01$) and serum TNF-α concentration ($r=0.55$, $p<0.01$). These results suggest that SCFAs and TMAO may be key mediators in the regulation of thrombosis and inflammation by LP and CLP, and their changes may explain the in vivo anti-thrombotic effect of the combination despite the in vitro increase in MPA/PRI.

Intestinal Barrier Function

Immunofluorescence showed that the fluorescence intensity of ZO-1 and Occludin in LP-treated groups was higher than that in the control group, while their expression was downregulated in the CLP group (Figure 4A). Further fluorescent quantitative PCR results also showed significantly increased ZO-1 and Occludin mRNA in LP-treated groups, while the CLP group showed a significant decrease compared with the control group (Figure 4B). Fluorescent quantitative PCR

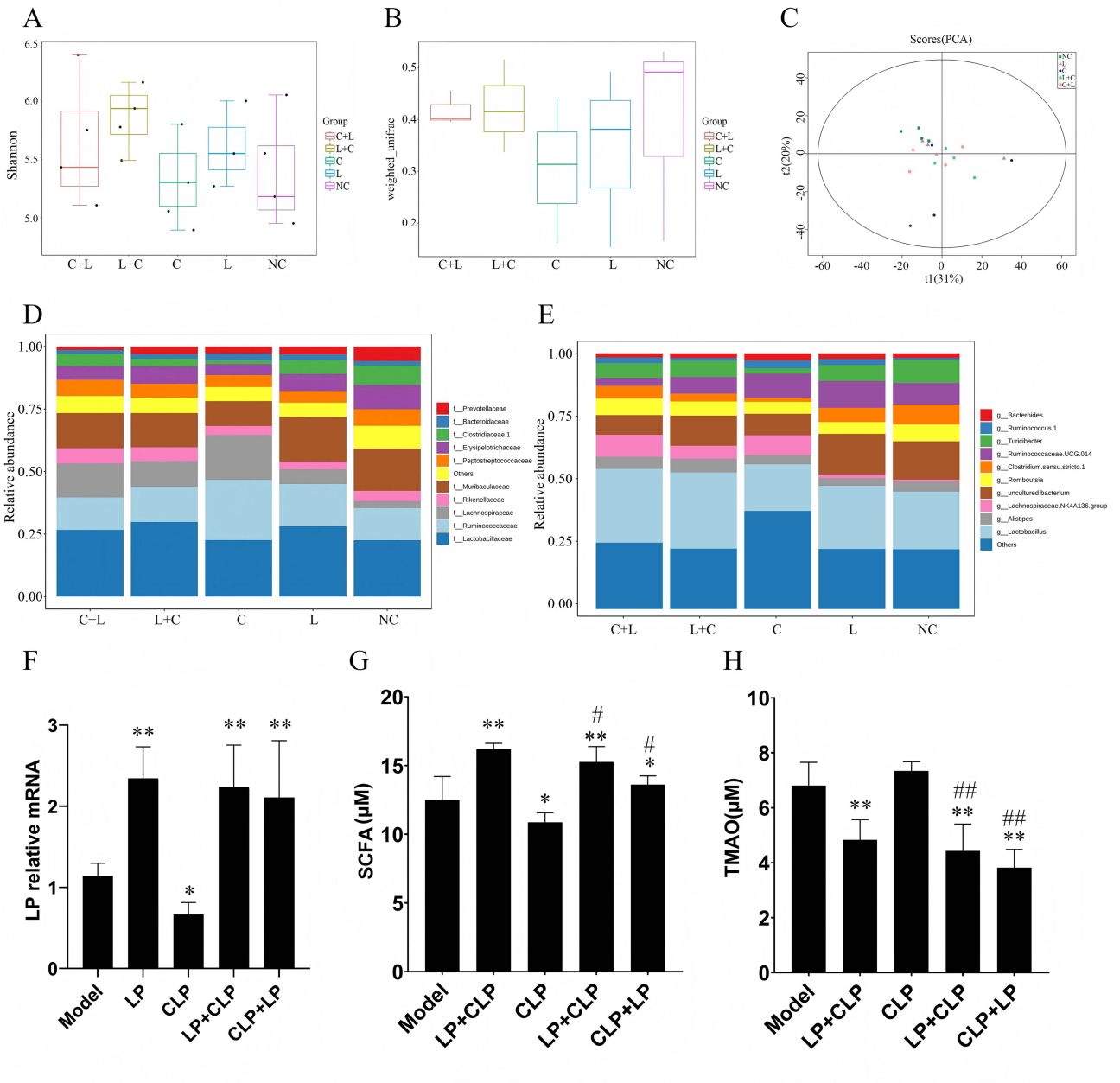

Figure 3 Effects of combined LP and CLP on gut microbiota structure (n=4). (**A**) Alpha diversity of gut microbiota (Shannon index), reflecting species richness and evenness; (**B**) Beta diversity of gut microbiota, showing inter-group community similarity; (**C**) Principal component analysis (PCA) based on beta diversity, showing community separation among groups; (**D** and **E**) Microbial community structure and composition at the family and genus levels (top 10 taxa by relative abundance); (**F**) Fluorescent quantitative PCR detection of LP colonization in the intestine; (**G** and **H**) Serum SCFA and TMAO concentrations. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test with Bonferroni correction. *$p < 0.05$ and **$p < 0.01$ vs Control; # $p < 0.05$ and ## $p < 0.01$ vs CLP.

detection of intestinal P-gp showed significantly increased P-gp mRNA in all groups, with further increased expression after combination treatment compared with the CLP group (Figure 4C). These results suggest that LP improves intestinal barrier function, and the combination therapy enhances this function, reducing the entry of harmful substances into the bloodstream and decreasing the risk of inflammatory responses and platelet activation.

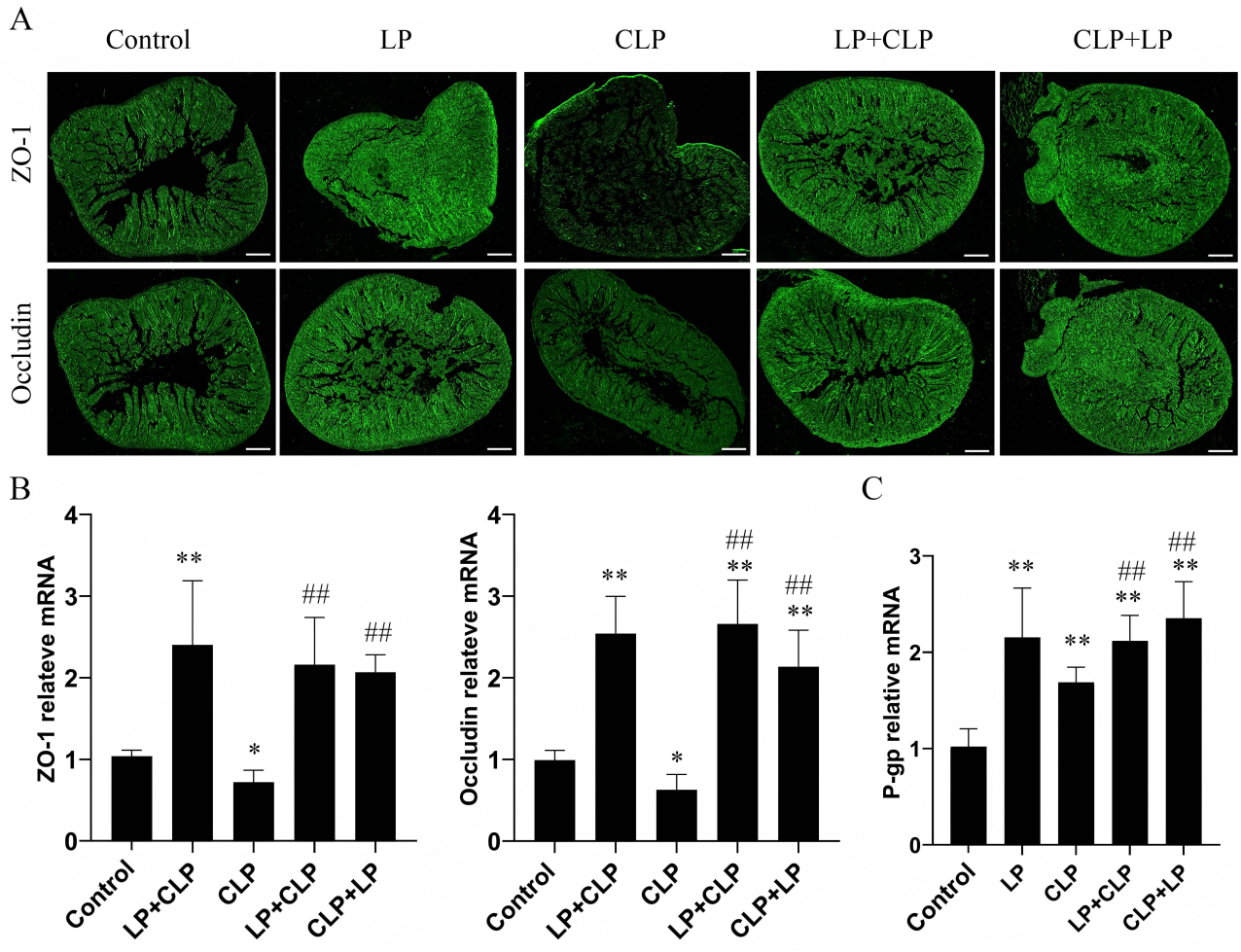

Figure 4 Protective effects of combined LP and CLP on intestinal barrier function (n=6). (**A**) Immunofluorescence detection of ZO-1 and Occludin expression (scale bar: 200 μm); (**B**) Fluorescent quantitative PCR detection of ZO-1 and Occludin mRNA expression; (**C**) Fluorescent quantitative PCR detection of P-gp mRNA expression. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test with Bonferroni correction. *p < 0.05 and **p < 0.01 vs Control; ## p < 0.01 vs CLP.

Discussion

Several studies using animal experiments and in vitro cell experiments have found that gut microbiota can participate in drug metabolism by expressing specific enzymes such as β-glucuronidase and nitroreductase, altering the chemical structure of drugs, and thus influencing their activity and efficacy.[8] In a study on antibiotics, certain strains of gut microbiota were found to hydrolyze or modify antibiotics, reducing their antibacterial activity and affecting treatment outcomes.[9]

Our results showed significant changes in blood drug concentrations across groups after 14 days of continuous culture in rats. Compared with CLP alone, combined treatment with LP and CLP significantly decreased blood drug concentrations, while MPA and PRI both increased significantly. This suggests that the colonization and functional activity of LP in vivo require a certain period and may partially reduce drug efficacy. However, compared with CLP alone, combined treatment with LP and CLP significantly improved the rate and quantity of thrombus formation and significantly inhibited the expression of inflammatory factors. P-selectin and GPIIb/IIIa are molecular markers of platelet activation and play important roles in thrombus formation.[10] P-selectin mediates the rolling of blood cells on the surface of endothelial cells and the adhesion of granulocytes and monocytes to platelets.[11] Circulating P-selectin mainly originates from vascular endothelial cells, so its levels increase during endothelial injury. On the other hand, after endothelial injury, high plasma levels of vWF mediate platelet binding to subendothelial collagen, initiate platelet aggregation by activating GPIIb/IIIa to

bind with fibrin, and promote thrombus formation. During thrombus formation, the fibrinolytic system is secondarily activated, leading to increased levels of D-dimer, a degradation product of cross-linked fibrin. Our experimental results showed that combined medication significantly inhibited P-selectin, GPIIb/IIIa, and D-dimer levels compared with CLP alone, suggesting that thrombus formation is inhibited by suppressing platelet activation.

It should be noted that there seems to be a discrepancy between the changes in platelet function indices (MPA and PRI) and in vivo thrombosis outcomes. MPA and PRI mainly reflect the direct response of platelets to specific agonists (ADP in this study) in vitro, while in vivo thrombosis is regulated by multiple factors such as vascular endothelial function, inflammatory response, and coagulation system balance. Although LP may slightly affect CLP metabolism (leading to decreased AM/CA and increased MPA/PRI), it can significantly improve vascular endothelial function and inhibit systemic inflammatory response by increasing SCFAs and decreasing TMAO. These effects may outweigh the slight reduction in direct anti-platelet efficacy of CLP, ultimately achieving a combined inhibitory effect on in vivo thrombosis. Future studies can further verify this hypothesis by using flow cytometry to detect platelet activation markers (such as integrin activation and alpha-granule secretion) and investigating the effects of SCFAs and TMAO on non-platelet cells (such as endothelial cells and white blood cells).

Our results show that LP has dual effects on the anti-thrombotic effect of CLP. On the one hand, LP may slightly reduce the metabolic activation of CLP, leading to decreased blood concentrations of AM and CA, and thus slightly increased MPA and PRI (reflecting weakened direct anti-platelet effect in vitro). On the other hand, LP can regulate gut microbiota to increase SCFAs and decrease TMAO, which in turn protect the intestinal mucosal barrier, inhibit systemic inflammatory response, and improve vascular endothelial function. These latter effects may play a more important role in in vivo thrombosis inhibition, thereby achieving a cooperative effect with CLP in reducing thrombosis, despite the slight increase in MPA and PRI.

Moreover, our results showed that gut microbial diversity in the LP group was higher than that in the Control group. The combined use of LP and CLP increased α and β diversity in the CLP-treated group. Additionally, LP effectively colonized the intestine, promoted SCFA release, and inhibited TMAO release. SCFAs can inhibit platelet activation and aggregation by regulating intracellular signaling pathways in platelets, such as inhibiting the degradation of cyclic adenosine monophosphate (cAMP) and increasing intracellular cAMP levels.[12] Furthermore, studies have found that butyric acid can upregulate the expression of tight junction proteins ZO-1, Occludin, and Claudin-1 in intestinal epithelial cells, thereby enhancing tight junctions between intestinal epithelial cells and reducing intestinal permeability.[13] We confirmed using immunofluorescence and real-time PCR that intestinal barrier function was enhanced after LP treatment. When intestinal barrier function is impaired, harmful substances in the intestine (such as endotoxins and bacteria) may enter the bloodstream, triggering systemic inflammatory responses. P-gp contributes to the processing of toxic metabolites,[14] and our results similarly showed that LP promoted the expression of intestinal mucosal P-gp. A limitation of this study is that we only detected the mRNA expression level of P-gp using RT-qPCR, but did not verify its protein level or functional efflux activity. Future studies can supplement these experiments to more comprehensively evaluate the effect of LP on P-gp expression and function.

SCFAs can also act on receptors on the surface of immune cells (such as macrophages, T cells, and B cells), such as G protein-coupled receptors 41 (GPR41), 43 (GPR43), and 109A (GPR109A),[15] activating intracellular signaling pathways to regulate immune cell functions, inhibit macrophages from producing inflammatory factors TNF-α and IL-1β, and promote the production of anti-inflammatory factor IL-10, thereby exerting anti-inflammatory effects.[16] Our results similarly showed that combined medication inhibited the release of inflammatory factors.TMAO, an important microbial metabolite, is closely associated with the occurrence and development of cardiovascular diseases. After entering the bloodstream, TMAO affects platelet function through multiple pathways, promotes platelet activation and aggregation, upregulates P-selectin expression on platelet surfaces, enhances platelet adhesion to endothelial cells, and further promotes thrombus formation.[17,18]

From a translational perspective, the combined use of LP and CLP may provide a new strategy for improving the anti-thrombotic effect of CLP in clinical practice, especially for patients with gut microbiota dysbiosis. However, it is also necessary to pay attention to the potential risks: the decrease in AM and CA concentrations suggests that LP may affect the pharmacokinetics of CLP by regulating drug transporters (such as P-gp) or metabolic enzymes, which may lead to

individual differences in the efficacy of CLP in humans. Therefore, in future clinical applications, it is necessary to monitor the blood concentration of CLP and adjust the dosage according to individual conditions to ensure the safety and efficacy of the combined treatment. Future studies can further explore the effects of LP and CLP on platelet function by detecting VASP-P (a specific marker of platelet activation) and performing platelet aggregation experiments with multiple agonists (such as ADP, collagen, and arachidonic acid), which can help clarify the pathway-specific effects of the combined treatment and their changes over time.

Despite significant research achievements in the interactions between gut microbiota and drug metabolism, the effects of microbial metabolites on platelet activation, and combined medication regimens, several limitations remain. First, the mechanisms underlying the interactions between gut microbiota and drug metabolism are not fully understood, particularly the dynamic changes and mutual influences between gut microbiota and drugs in the complex human physiological environment, which require further investigation. Second, although some key metabolites and signaling pathways involved in the regulation of platelet activation by microbial metabolites have been identified, the interconnections and synergistic effects between these mechanisms remain unclear.

Conclusion

Lactobacillus paracasei can regulate the structure of gut microbiota, including increasing SCFA-producing bacteria and inhibiting TMAO-producing bacteria. This regulation can protect the intestinal mucosal barrier and inhibit inflammatory responses, thereby cooperatively acting with CLP to inhibit in vivo thrombosis and improve coagulation function. Although the combined treatment leads to a slight increase in MPA and PRI (possibly due to decreased CLP metabolites) and the specific mechanism needs further verification, the overall results suggest that the combined use of LP and CLP has potential value in anti-thrombotic therapy. Future studies should focus on verifying the causal relationship between gut microbiota metabolites and anti-thrombotic effects, as well as exploring the safety and efficacy of this combination in humans.

Statement of Data Availability

The analyzed data involved in this study are available from the corresponding author upon reasonable request.

Ethical Approval and Consent to Participate

The study was approved by the Institutional Ethics Committee of Shandong Provincial Third Hospital (No: 2024-145SP2H) and was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Research Council, USA.

Author Contributions

Both authors made a significant contribution to the work reported, Xiaona Ren is in the conception, study design, execution, acquisition of data, analysis and interpretation; Caicai Liu took part in drafting, revising or critically reviewing the article; Both authors gave final approval of the version to be published and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

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