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Distinct Microbial Signatures and their Predictive Value in Recurrent Acute Pancreatitis: Insights from 5-Region, 16S rRNA Gene Sequencing

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Research Article

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ABSTRACT

Background: The Recurrent Acute Pancreatitis (RAP) poses significant clinical challenges and the underlying microbial factors contributing to RAP remain poorly understood. This study aims to identify the microbial profiles associated with RAP and explore the potential microbial predictors for RAP.

Methods: Ninety patients were classified into Non-Recurrent Acute Pancreatitis (NRAP) (n=68) and RAP (n=22) groups based on the number of pancreatitis episodes. Clinical characteristics were documented and the microbial composition of serum samples was analysed using 5-Region (5R), 16S rRNA gene sequencing. Key microbial taxa and functional predictions were made. Additionally, a random forest model was used to assess the predictive value of microbial features for RAP. The impact of *Staphylococcus hominis* on RAP was further evaluated in an experimental mouse model.

Results: Microbial analysis revealed specific taxa were differentially abundant between the groups. Linear discriminant analysis effect size (LefSE) analysis highlighted significant microbial difference with *Paracoccus aminovorans*, *Corynebacterium glucuronolyticum* and *Staphylococcus hominis* being prominent in RAP. Functional predictions indicated enrichment of metabolic pathways in the RAP group. Random forest analysis identified key microbial taxa with an Area Under the Curve (AUC) value of 0.759 for predicting RAP. Experimental validation showed that *Staphylococcus hominis* exacerbates pancreatic inflammation in mice.

Conclusions: This study identifies distinct clinical and microbial features associated with RAP, emphasizing the role of specific bacterial taxa in pancreatitis recurrence. The findings suggest that microbial profiling could enhance the diagnosis and management of RAP, paving the way for personalized therapeutic approaches.

Keywords: Acute pancreatitis; Recurrent acute pancreatitis; Microbiome; 16S rRNA gene sequencing; *Staphylococcus hominis*

INTRODUCTION

Acute Pancreatitis (AP) is an acute inflammation of the pancreas caused by etiologic factors such as gallstones, alcohol use and hypertriglyceridemia that can lead to significant morbidity and mortality [1]. The incidence of AP is estimated at 13 cases to 49 cases per 100,000 persons per year [2]. Despite advances in medical care, the recurrence of AP following an index attack remains high with a 11%~36% risk of developing Recurrent Acute Pancreatitis (RAP) [3,4]. The progression from AP to RAP to eventual Chronic Pancreatitis (CP) is a disease continuum [5]. RAP is associated with a higher risk of complications, developing chronic pancreatitis and diminished quality of life, underscoring the need for better understanding of its pathogenesis and early prediction [6].

Identified risk factors for RAP include ongoing alcohol use, smoking, genetic predispositions and certain contributing metabolic factors like hypertriglyceridemia and hypercalcemia. However, some RAP patients have no clear pathogenic factor, which is clinically challenging and has substantial socioeconomic burdens. Recent studies reported a rate of 4%~32.3% of RAP cases developed chronic diseases [7-10]. Appropriate triage, close monitoring, accurate prediction and early intervention can prevent recurrent AP attacks. Thus, early identification of AP patients at high risk of developing RAP is essential.

Recent decades have witnessed that the changes of gut microflora has been implicated in the development and progression of pancreatitis. For instance, AP patients had a higher relative abundance of potentially pathogenic bacteria and a lower relative abundance of beneficial bacteria when compared with healthy controls, with relevance to disease severity and poor prognosis [11,12]. One proposed mechanism is the translocation of enteric bacteria into peripheral circulation, which can trigger systemic inflammation and exacerbate pancreatic injury [13,14]. In addition, researches have reported that there is an authentic microbiome in human blood, leading to the development of several chronic diseases [15,16]. The discovery of blood microbiome may serve as a critical link between gut dysbiosis and the inflammatory processes in pancreatitis.

In light of these findings, our study aims to investigate the potential involvement of the blood microbiome in the development of RAP using 5-Region (5R), 16S rDNA sequencing. We collected serum samples from patients within 24 h of the onset of AP and divided patients into NRAP and RAP groups based on medical history. Our objective was to explore the biological and clinical significance of blood microbiome that could aid in the early prediction and diagnosis of RAP, thereby facilitating the development of more effective diagnostic models and therapeutic strategies.

MATERIALS AND METHODS

Human sample collection

This was a single-center, cross-sectional study. Patients were enrolled in the First Affiliated Hospital of Naval Medical University, Shanghai, China, between September 2022 and April 2023. Serum samples were collected from patients diagnosed with AP according to the diagnostic criteria within 24 h of disease onset [1]. All participants provided informed consent. According to the diagnostic criteria for RAP, which includes clinical symptoms, serological and imaging tests confirming AP, with ≥ 2 episodes, no permanent histological changes such as exocrine or endocrine dysfunction, fibrosis or calcification and an interval of ≥ 3 months between the two AP episodes, AP patients were further classified into NRAP and RAP groups [6]. The major exclusion criteria were chronic pancreatitis, inflammatory bowel disease, immunosuppressive disease, cancer, the use of antibiotics within two months of enrolment. Detailed demographic and clinical information, including age, gender and medical history and laboratory findings were

recorded for each patient. Ethical approval was obtained from the Ethics Committee. The samples were stored at -80°C refrigerator before analysis.

DNA extraction and 5R 16S rRNA gene sequencing

DNA was extracted from serum samples using standardized protocols to ensure high-quality genetic material. 5-Region (5R), 16S rRNA sequencing was used in the study to mitigate host DNA interference and facilitate microbiota detection in samples with low microbial content but high host proportions. The experiment utilized Polymerase Chain Reaction (PCR) to amplify variable regions of the 16S rRNA gene (V2, V3, V5, V6 and V8). The purified PCR products were evaluated using an Agilent 2100 Bioanalyzer (Agilent, USA) and the library quantification kit from Illumina (Kapa Biosciences, Woburn, MA, USA) ensuring that the qualified library concentration was above 0.3 ng/μL. Paired-end sequencing was performed using the Illumina NovaSeq 6000 sequencing platform in the PE150 sequencing mode.

Bioinformatics analysis and visualization

The sequencing data was analysed using the Short Multiple Regions Framework (SMURF) analysis pipeline, employing the optimized Greengenes (May 2013 version) database. The purified Amplicon Sequence Variants (ASVs) were generated to identify microbial profiles. Alpha diversity analysis was conducted based on the resulting species-level abundance tables, using indices such as observed species, Shannon, Simpson, Chao1 and goods coverage to evaluate intra-sample diversity. Beta diversity was assessed by calculating two distances (Bray-Curtis) and performing six analyses to evaluate inter-sample/group diversity. A Venn diagram showing overlapping ASVs between the two groups was created using the VennDiagram package in R (version 4.3.1). The ggplot2 package was used for visualization. Linear discriminant analysis effect size (LefSE) was used to compare microbial compositions with thresholds set at an LDA score>3 and $p<0.05$.

Microbiota function prediction and clinical correlation analysis

The functional potential of the microbiota was predicted using PICRUSt2 software. The predicted functional profiles were analysed for differences using Statistical Analysis of Metagenomic Profiles (STAMP) (version 2.1.3). Correlation analysis between clinical information and microbiota was performed using Spearman's rank correlation coefficient and then visualized using the pheatmap package (Version 1.0.12), which assesses the strength and direction of association between clinical variables and microbial abundance.

Disease diagnostic model construction and validation

Based on the sequencing data, a random forest regression model for RAP was constructed with 70% of the samples assigned to the training cohort and the remaining 30% to the validation cohort. Ten-fold cross-validation was applied to the training cohort. The most important variables were used to build the predictive model and Receiver Operating Characteristic Curve (ROC) curves were calculated to distinguish between NRAP and RAP patients. The confidence intervals for the ROC curves were calculated using the pROC package in R.

Animal experiment

Male C57BL/6 mice (6 weeks-8 weeks old) obtained from GemPharmatech Co., Ltd., (Nanjing, China), were treated with broad-spectrum antibiotics (ampicillin 1 g/l, neomycin 1 g/l, metronidazole 1 g/l and vancomycin 0.5 g/l) in their drinking water for 4 weeks before experiment. After antibiotic treatment, mice were randomly divided into Phosphate Buffered Saline (PBS) gavaged group (n=8) and *Staphylococcus hominis* gavaged group (n=8). Mice were administered 200 ul PBS/mice only or at a dose of 107 Colony-Forming Units (CFU) of *Staphylococcus hominis* intragastrically once every two days for 2 weeks. RAP was induced by 2 attacks of AP with 8 hourly injections of 100 ug/kg caerulein, after the first attack, mice were allowed to recover for 7 days [17]. At 24 h after the first injection of

caerulein in second period, mice were sacrificed and analyzed. All animal care and experimental protocols were approved by the Animal Ethics Committee of the First Affiliated Hospital of Naval Medical University, Shanghai, China.

Enzyme-Linked Immunosorbent Assays (ELISA)

Mice serum was obtained by centrifuging whole blood samples. Serum levels of Amylase (Abcam, ab102523), IL-1β (Abcam, ab197742) and TNF-α (Abcam, ab102523) was measured using commercial ELISA kits according to manufacturer's instructions.

Histological analysis

Formaldehyde fixed pancreas were embedded in paraffin, sectioned into slices and stained with Hematoxylin Eosin (HE). Pancreatic histological score was assessed under a light microscope (Olympus, Tokyo, Japan) according to Rongione's standard [18]. Inflammatory cells in pancreas were evaluated by detection of F4/80 by immunohistochemistry. Briefly, slides are heated in a 70ºC oven for 30 min and then deparaffinized in xylene, followed by rehydration through a graded ethanol series. After antigen repairing procedure, slides were incubated with F4/80 antibody (Abcam, ab300421, 1:500) at 4ºC overnight. After washing with PBS, slides were developed with DAB substrate and counterstained with hematoxylin. The Immunohistochemistry (IHC) positive staining was analysed by ImageJ (V1.8).

Flow cytometry

Pancreatic tissue samples were harvested and digested in collagenase IV solution at 37ºC for 30 min. Pancreatic cells were subsequently filtered through a 70 um cell strainer. After washing and lysis of erythrocytes, single-cell suspensions were incubated for 15 min at room temperature in stain buffer with the following antibodies for surface markers: mCD45 (#103108, BioLegend, FITC conjugate), mCD11b (#101230, Biolegend, PerCP conjugate) and mF4/m80 (#123116, BioLegend, APC conjugate). Sample acquisition was carried out on Cytoflex flow cytometer (Beckman CytoFLEX S, USA) and data were analysed using FlowJo 10.8 (BD Biosciences, San Jose, CA).

Statistical analysis

Clinical characteristics of the two patient groups were analysed using SPSS software (version 19.0, IBM Corp). For normally distributed continuous variables, data were presented as mean ± Standard Deviation (SD); for non-normally distributed continuous variables, data were presented as median (interquartile range (IQR)). Categorical variables were presented as numbers (percentages). The p-value for categorical variables was calculated using the chi-square test or Fisher's exact test. Continuous variables were analysed using the t-test or the non-parametric Kruskal-Wallis test. A two-sided p-value of less than 0.05 was considered statistically significant.

RESULTS

Clinical baseline information

A total of 90 patients were included in this study, classified into 68 NRAP cases and 22 RAP cases based on the number of pancreatitis episodes. The demographic and clinical characteristics of the participants were summarized in Table 1. No significant differences were found among the key clinical variables, including age, gender and aetiology. The NRAP group showed higher levels of LDH compared to the RAP group (median 311 U/L, IQR (198-492) *vs.* median 219 U/L, IQR (155-281); p=0.021). Similarly, BUN levels were higher in the NRAP group than in the RAP group (median 5.1 mmol/L, IQR (4.0-6.6) *vs.* median 4.5 mmol/L, IQR (3.2-4.9); p=0.025). The proportion of Severe Acute Pancreatitis (SAP) patients was higher in the NRAP group compared to the RAP group (35.3% *vs.* 22.7%; p=0.138), but this difference was not statistically significant. The duration of hospitalization showed no significant difference between the two groups (NRAP: Median 10 days, IQR (5-21) *vs.* RAP: Median 8 days, IQR (5-12); p=0.171).

Table 1. Demographic and clinical characteristics of NRAP and RAP patients.

Factors	NRAP (n=68)	RAP (n=22)	p-value
Age (years), median (IQR)	48 (36-61)	51 (39-58)	0.735
Male gender, n (%)	38 (56)	15 (68)	0.311
Laboratory findings			
Triglyceride, median (IQR), mmol/L	1.26 (0.64-3.81)	1.58 (0.81-3.99)	0.866
CRP, median (IQR), mg/L	209.0 (73.7-305.5)	170.0 (83.8-204.5)	0.381
Blood amylase, median (IQR), U/L	503 (237-1047)	188 (81-365)	0.055
Blood lipase, median (IQR), U/L	278.7 (153.9-409.5)	111.4 (72.8-286.7)	0.254
LDH, median (IQR), U/L	311 (198 -492)	219 (155-281)	0.021
BUN, median (IQR), mmol/L	5.1 (4.0-6.6)	4.5 (3.2-4.9)	0.025
ALT, median (IQR), U/L	26 (15-75)	19 (15-30)	0.078
Scr, median (IQR), umol/L	64.5 (56.0-88.0)	62.0 (50.0-74.0)	0.270
Hospital stay (days), median (IQR)	10 (5-21)	8 (5-12)	0.171
Disease severity, n (%)			
MAP	21 (30.9)	11 (50.0)	0.105
MSAP	23 (33.8)	6 (27.3)	0.570
SAP	24 (35.3)	5 (22.7)	0.276
Etiology, n (%)			
Biliary	31 (45.6)	6 (27.3)	0.131
Hypertriglyceridemia	17 (25.0)	9 (40.9)	0.155
Alcohol consumption	4 (5.9)	1 (4.5)	0.813
Other	16 (23.5)	6 (27.3)	0.724

Note: CRP: C-Reactive Protein; LDH: Lactate Dehydrogenase; BUN: Blood Urea Nitrogen; ALT: Alanine aminotransferase; Scr: Serum creatinine; MAP: Mild Acute Pancreatitis; MSAP: Moderately Severe Acute Pancreatitis; SAP: Severe Acute Pancreatitis.

Microbial profile of NRAP and RAP patients

The microbial compositions of serum samples from NRAP and RAP patients were characterized by 5R 16S rRNA gene sequencing. The sequencing quality was eligible, with no samples discarded and an average reads count of 33,312 per sample. As shown in Figure 1A, there was no significant difference in α-diversity between the two groups, indicating that the overall composition of NRAP and RAP patients was similar. The species richness rarefaction curve gradually levelled off, indicating a reasonable number of individual samples (Figure 1B). NMDS and UPGMA cluster analysis based on the UniFrac algorithm showed no significant difference in microbial β-diversity between the two groups (Figures 1C and 1D). Venn diagrams showed the common and unique phyla and genera detected in the NRAP and RAP groups (Figures 1E and 1F).

Figure 1. Comparison of microbial diversity between NRAP and RAP patients. **Note:** (A) Alpha-diversity analysis; (B) Species richness rarefaction curve; (C) NMDS plot; (D) UPGMA clustering analysis; (E) Venn diagram of shared and unique bacterial phyla and genera in NRAP groups; (F) Venn diagram of shared and unique bacterial phyla and genera in RAP groups.

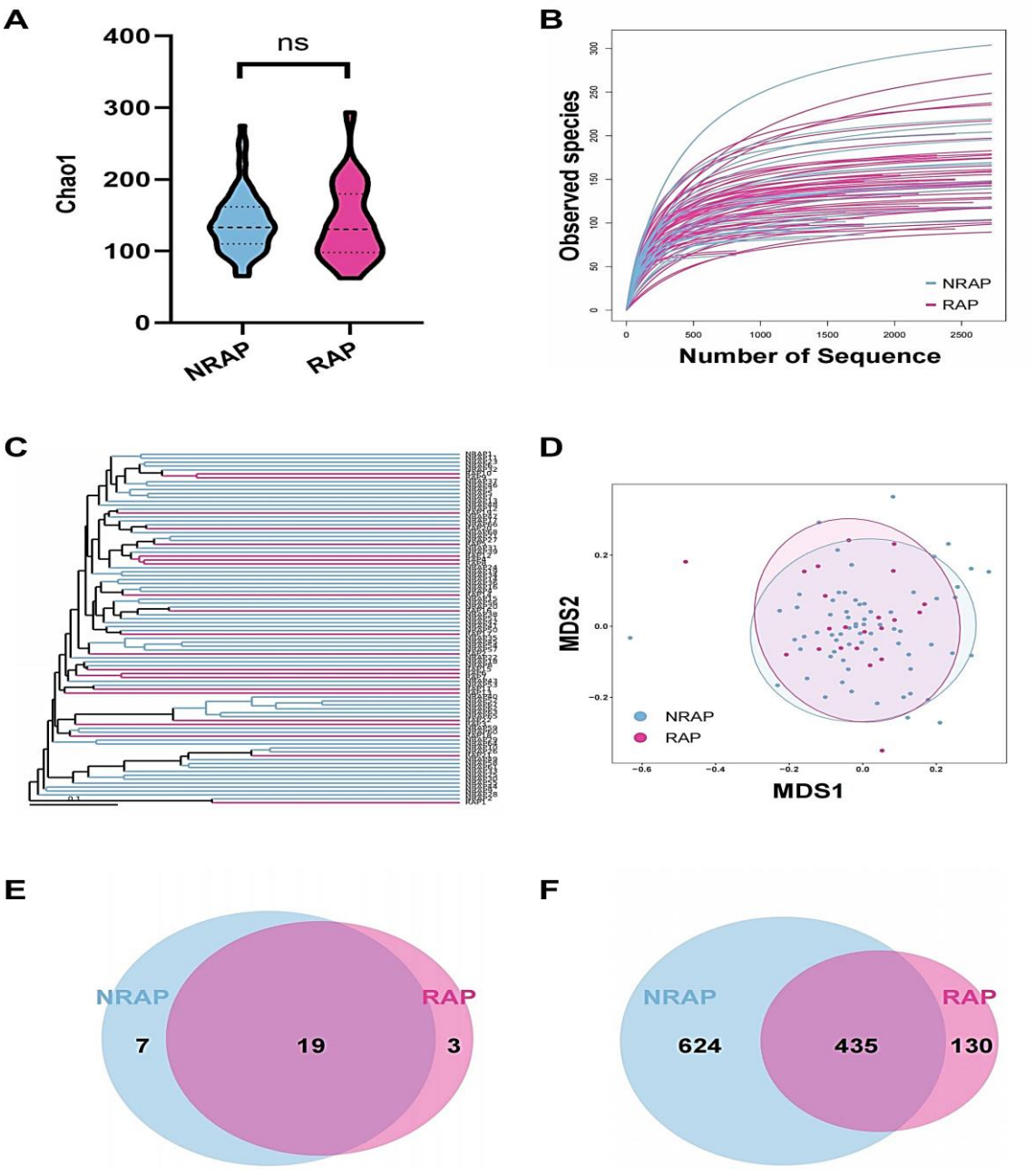
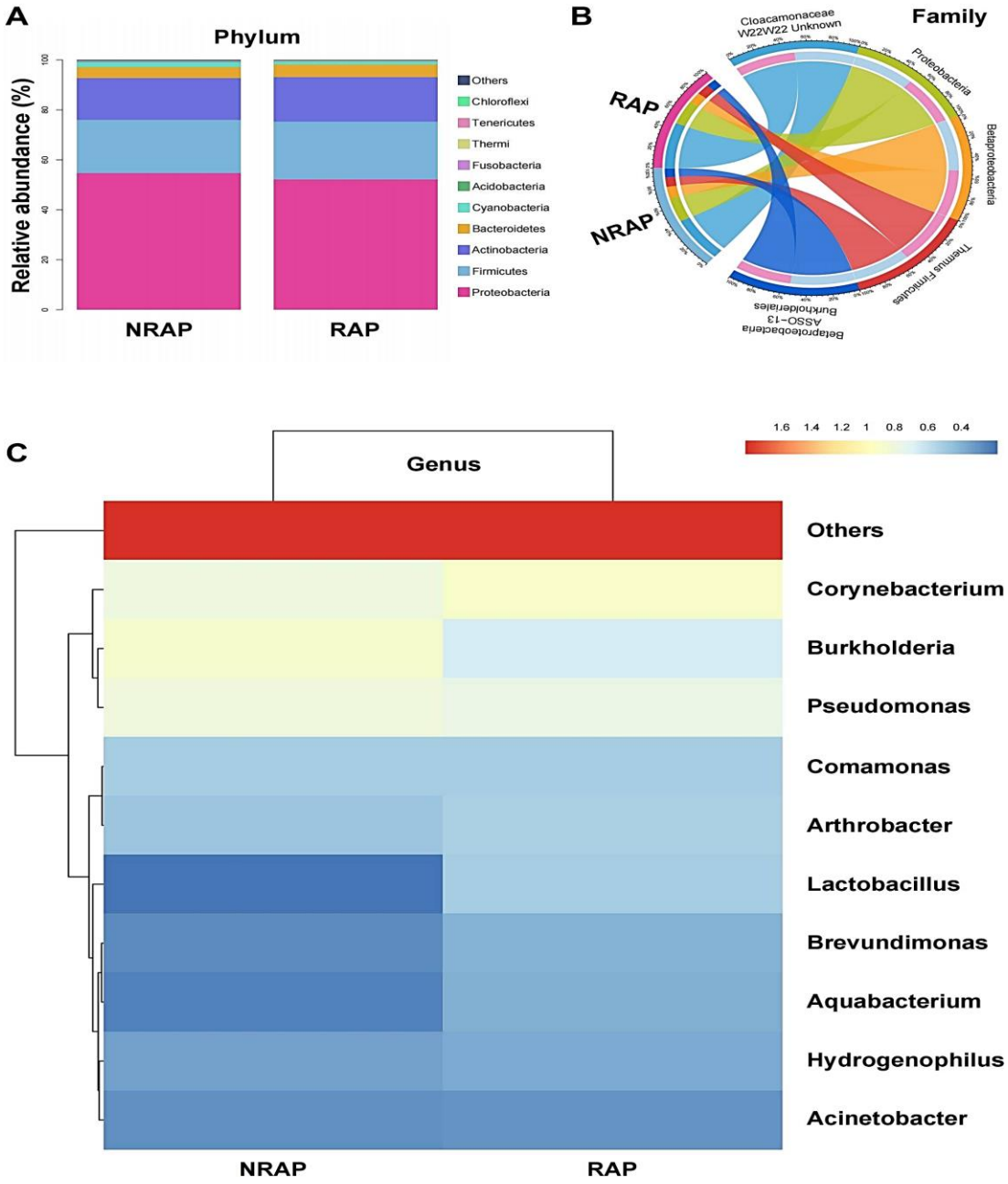


Figure 2A presents a stacked bar chart of the top 10 relative abundance bacterial species at the phylum level in the NRAP and RAP groups, mainly composed of *Proteobacteria*, *Firmicutes* and *Actinobacteria*. A chord diagram at the family level visualizes the microbial community composition and the associations between the two groups (Figure 2B). The top 10 genera profiles are shown in Figure 2C. *Burkholderia* was more frequent in the NRAP group than in the RAP group, while the relative abundance of *Corynebacterium* was higher in the RAP group. NRAP group showed a lower relative abundance of *Lactobacillus* when compared with the RAP group.

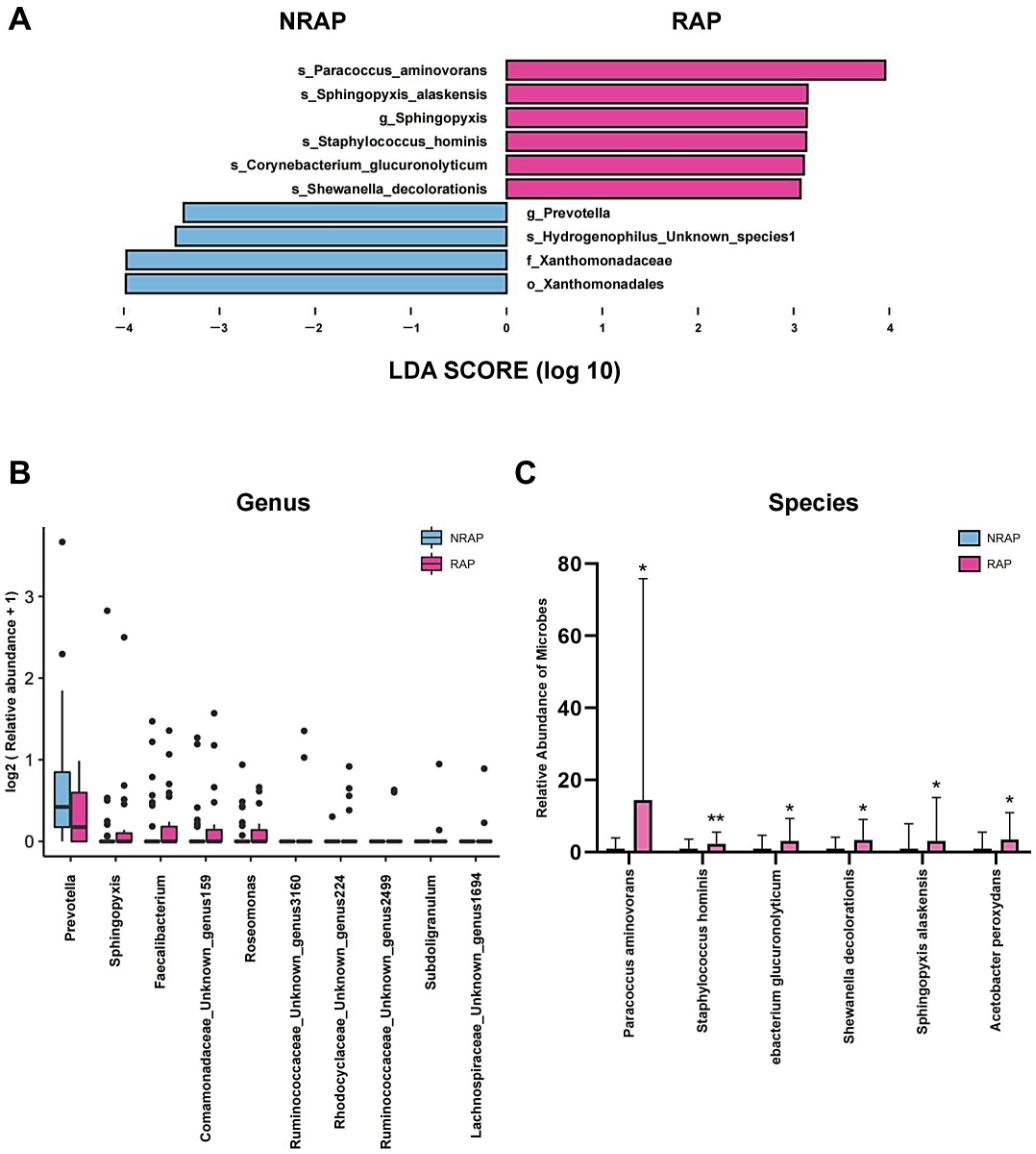
Figure 2. Relative abundance of major bacterial taxa in NRAP and RAP patients. **Note:** (A) Stacked bar chart of top 10 bacterial phyla in NRAP and RAP groups; (B) Chord diagram showing associations of microbial families between NRAP and RAP groups; (C) Top 10 genera profiles comparing NRAP and RAP groups.



Taxonomic characteristics of NRAP and RAP patients

LefSE analysis depicted significant differences in bacterial taxa between the NRAP and RAP groups (Figure 3A). The key microbial taxa in the NRAP group included g_*Prevotella*, f_*Xanthomonadaceae* and o_*Xanthomonadales*, whereas in the RAP group, g_*Sphingopyxis* was the dominant taxon. A boxplot visually displayed the top 10 ranking genera with significant differences (Figure 3B). At the species level, compared to the NRAP group, the RAP group showed higher abundances of *Paracoccus aminovorans* (2.172% *vs.* 0.150%, p=0.048), *Staphylococcus hominis* (0.503% *vs.* 0.210%, p=0.01) and *Corynebacterium glucuronolyticum* (0.436% *vs.* 0.140%, p=0.03) (Figure 3C).

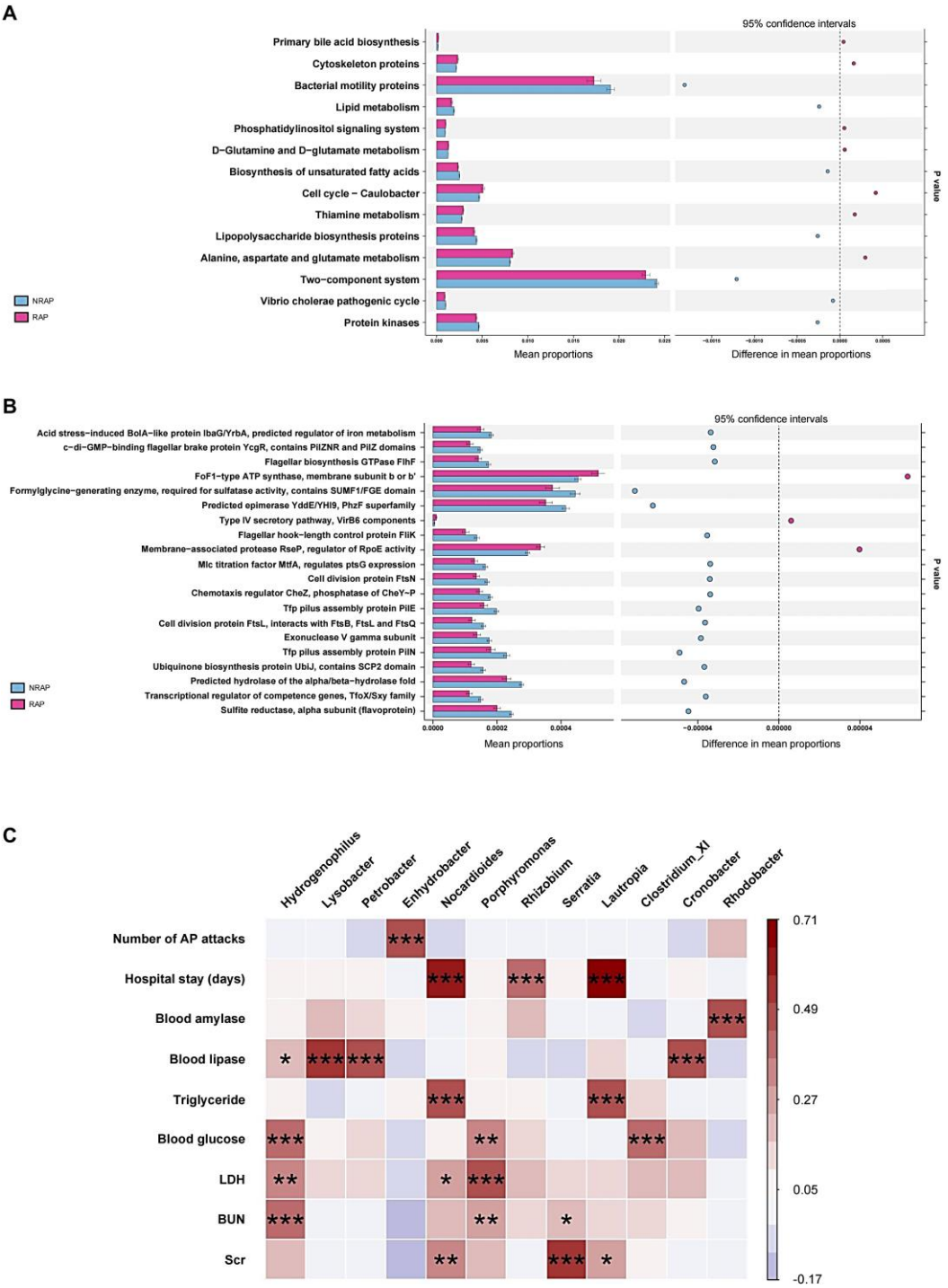
Figure 3. Differentially abundant taxa between NRAP and RAP patients. **Note:** (A) LefSE analysis; (B) Boxplot of top 10 genera with significant differences between NRAP and RAP groups; (C) Comparison of specific bacterial species abundances in NRAP and RAP groups.



Using PICRUSt analysis to predict gene functions of the microbiota, we found that the differential microbial community in the RAP group were enriched in KEGG pathways such as alanine, aspartate, glutamate metabolism, thiamine metabolism cell cycle-*caulobacter* and COG pathways such as FoF1-type ATP synthase, membrane subunit b or 'b' when compared to the NRAP group (Figures 4A and 4B).

Spearman correlation analysis was conducted to identify associations between blood microbiota and clinical indicators, including demographic characteristics, laboratory tests, duration of hospitalization and number of pancreatitis episodes (Figure 4C). At the genus level, *Enhydrobacter* was positively correlated with the number of AP attacks ($r=0.45$, $p<0.001$). *Lautropia* showed a strong positive correlation with hospitalization duration ($r=0.71$, $p<0.001$) and a moderate positive correlation with TG ($r=0.48$, $p<0.001$). Additionally, *Nocardioides* was moderately positively correlated with both hospitalization duration and TG, with statistical significance ($r=0.58$, $p<0.001$; $r=0.47$, $p<0.001$).

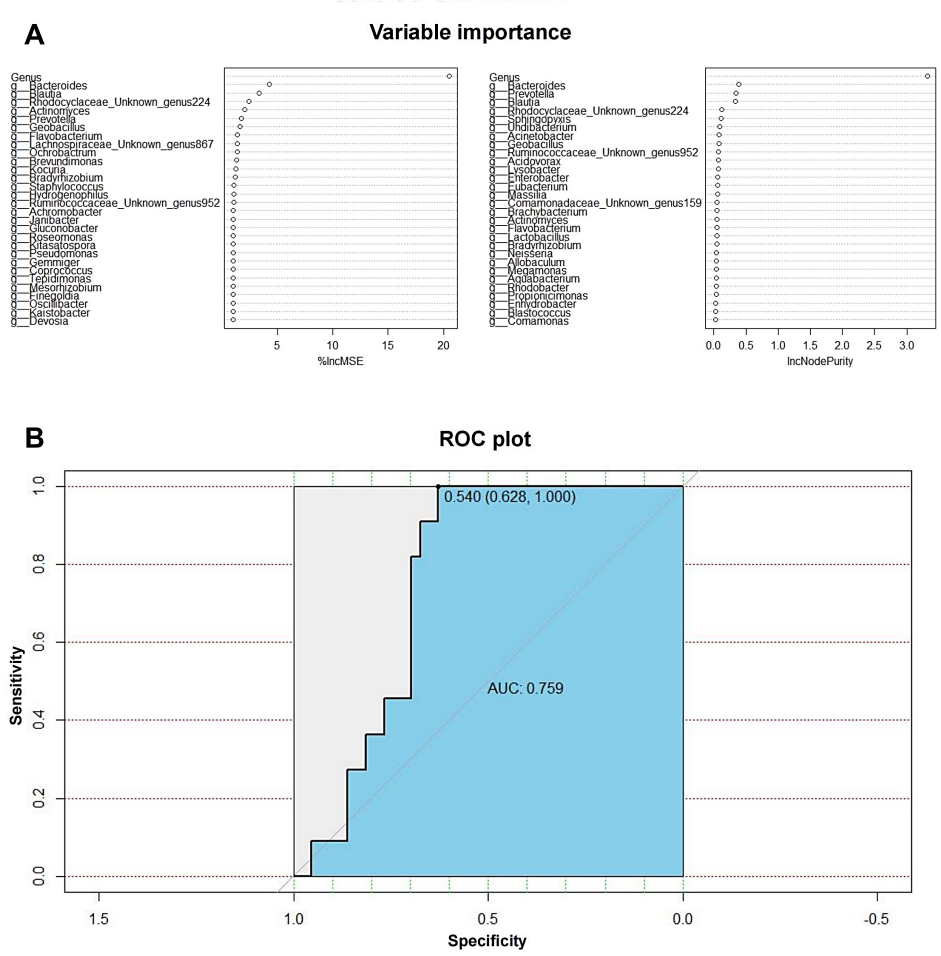
Figure 4. Functional predictions and clinical correlations of blood microbiota in NRAP and RAP patients. **Note:** (A) KEGG pathway enrichment; (B) COG pathway enrichment; (C) Spearman correlation heatmap showing associations between microbiota and clinical indicators.



Potential microbial features predictive of RAP

To investigate the value of blood microbial features in predicting RAP, we used random forest to identify taxa associated with RAP. The IncMSE and IncNodePurity analyses revealed the differing contributions of various genera (Figure 5A). In the validation cohort, the diagnostic model constructed using the random forest algorithm had an AUC value of 0.759 (95% CI: 0.6346-0.8834, cutoff>0.540, sensitivity: 100%, specificity: 62.8%), suggesting the potential of serum microbiota to predict RAP (Figure 5B).

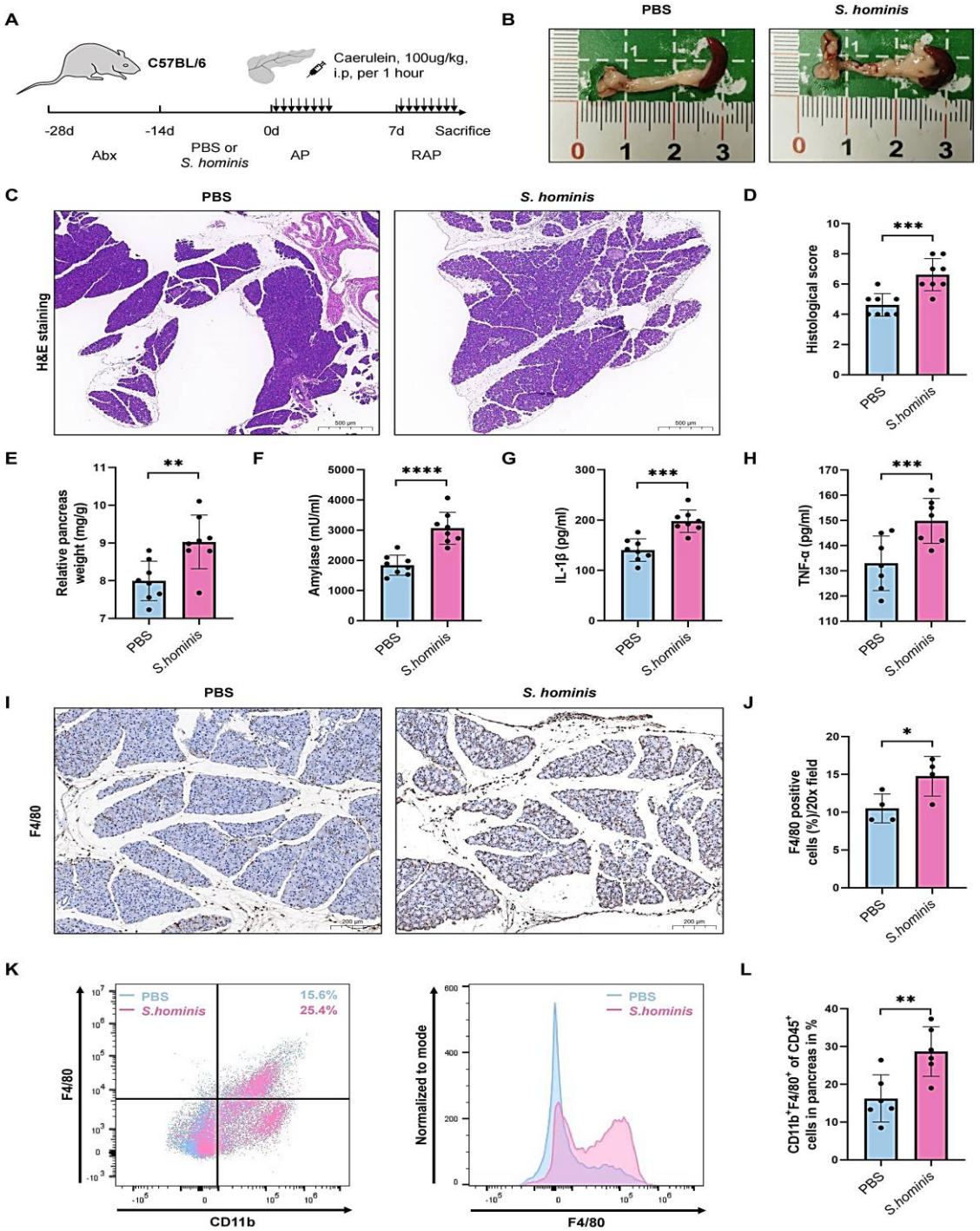
Figure 5. Predictive value of microbial features for RAP diagnosis. **Note:** (A) IncMSE and IncNodePurity analysis from the random forest model identifying key taxa for RAP prediction; (B) ROC curve of the diagnostic model with an AUC value of 0.759 for RAP prediction.



Staphylococcus hominis exacerbates experimental rap in mice

To further validate the hypothesis that microbes associated with RAP can participate in disease progression, *Staphylococcus hominis*, a gram-positive bacterium from the *Staphylococcus* genus, was selected in this study based on the LefSE analysis (Figure 3A), the top five differential species data between the two groups (Figure 3C) and previous literature reports. Antibiotic-treated, microbiota-depleted mice were gavaged with *Staphylococcus hominis* or PBS as control for two weeks and then induced with caerulein to create an RAP model (Figure 6A). The relative pancreatic weight ratio in the *Staphylococcus hominis* group was higher than in the PBS group, indicating more severe pancreatic inflammation (Figures 6B and 6E). Histological evaluation of the pancreas showed more edema, significant inflammatory cell infiltration and higher pancreatic tissue scores in the *Staphylococcus hominis* group compared to the PBS group (Figures 6C and 6D). Serum biochemical analysis revealed elevated levels of serum amylase, pro-inflammatory cytokines TNF-α and IL-1β in the *Staphylococcus hominis* group compared to the PBS group (Figures 6F-6H). Immunohistochemical detection using F4/80 identified increased macrophage infiltration in the pancreas of the *Staphylococcus hominis* group compared to the PBS group (Figures 6I and 6J). Flow cytometry further confirmed increased macrophage infiltration in the pancreas of the *Staphylococcus hominis* group relative to the PBS group (Figures 6K and 6L).

Figure 6. Impact of *Staphylococcus hominis* on pancreatitis severity in a mouse RAP model. **Note:** (A) Experimental design for PBS or *Staphylococcus hominis*-gavaged mice RAP models; (B) Relative pancreatic weight ratio in RAP models; (C) H&E staining of pancreatic tissues to assess histological damage; (D) Histological scores of pancreatic tissues based on H&E staining; (E) Serum amylase levels in PBS or *Staphylococcus hominis*-gavaged mice; (F) Serum TNF-α levels in PBS or *Staphylococcus hominis*-gavaged mice; (G) Serum IL-1β levels in PBS or *Staphylococcus hominis*-gavaged mice; (H) Immunohistochemical detection of macrophage infiltration in the pancreas; (I) Quantification of CD11b+F4/80+ macrophage infiltration in the pancreas *via* flow cytometry; (J) Representative images of pancreatic tissue histology (H&E staining); (K) Flow cytometry analysis of immune cell populations in the pancreas; (L) Correlation between serum cytokine levels and histological scores.



DISCUSSION

Approximately 11%-36% of AP cases will face recurring attacks after fully recover. In up to 20% of recurring cases, the cause remains unclear. A nationwide population-based cohort study reported that transition incidence rates to CP as 12.1 (95% CI, 8.1-18.1) from AP and 46.8 (95% CI, 31.6-69.3) from RAP, indicating a markedly higher risk of CP and eventually Pancreatic Cancer (PC) in patients with RAP [19]. Therefore, studying RAP is essential for the entire disease spectrum. Currently, no studies so far have reported on the contributions of pathogens to RAP, highlighting the necessity of our research. In this study, we comprehensively investigated the relationship between blood microbes and RAP by 5R 16S rRNA sequencing and observed the differences in the microbial profiles between NRAP and RAP, and the ability of the specific pathogen to exacerbate pancreatitis in mice, providing new insights into the underlying contributing factors of RAP.

The clinical characteristics of the NRAP and RAP groups revealed subtle differences. Bioinformatics analysis was performed to examine microbial diversity, richness and community structure between NRAP and RAP groups. The analysis showed no significant differences in overall diversity but identified specific taxa differences. The RAP group exhibited higher abundances of g_*Corynebacterium*, g_*Pseudomonas* and other important genuses. The RAP group exhibited higher abundances of *Paracoccus aminovorans*, *Staphylococcus hominis* and *Corynebacterium glucuronolyticum*. LefSE analysis further highlighted key microbial taxa between the groups, with g_*Prevotella* dominant in NRAP, while g_*Sphingopyxis* was prevalent in RAP. These taxa may serve as potential biomarkers for RAP.

Corynebacterium species, club-shaped gram-positive rods, are commonly found in animal hosts and are part of healthy human skin flora. Recently, they have recently been identified as causative agents of severe bloodstream infections, infective endocarditis, pneumonia and meningitis [20-23]. Furthermore, growing evidence suggests that *Corynebacterium* species act as opportunistic pathogens in long-term hospitalized patients and have developed to drug resistance [24,25]. The predominance of *Corynebacterium* species in the RAP group suggests that they may be high-risk pathogens. Notably, *Corynebacterium glucuronolyticum* was dominant in the RAP group; this rare isolate has only recently been recognized as a potential urogenital pathogen [26]. Its influence on inflammatory diseases is still not well documented, with few bibliographical references available. *Pseudomonas* is a widespread, saprophytic bacterium in the environment, consisting of more than 200 species. Among these, *Pseudomonas aeruginosa* is well-known for causing acute or chronic infections in immunocompromised individuals and poses significant treatment challenges due to its rapid acquisition of drug resistance. In our study, *Paracoccus aminovorans* was highlighted by LefSE analysis as a top-ranked differential species between the two groups. However, there is currently no evidence linking *Paracoccus aminovorans* to any diseases and further research is needed to explore this potential association. Functional predictions indicated enrichment of metabolic pathways and cell cycle regulation in the RAP group, linking microbial functions to disease progression by influencing the metabolic and proliferative processes. Significant correlations between specific genera and clinical indicators, such as *Enhydrobacter* with AP attacks and *Lautropia* with hospitalization duration, were observed. The random forest analysis provided a robust model for predicting RAP using microbial features, demonstrating an AUC value of 0.759 coupled with high sensitivity. This underscores the potential for blood microbial profiling in clinical settings to predict and manage RAP effectively.

Staphylococcus hominis, another dominant species in the RAP group, is the second most commonly isolated coagulase-negative *Staphylococcus* species (CoNS) from normal human skin [27]. Conversely, it has been reported to be an opportunistic pathogenic bacterium and may cause bloodstream infections, endocarditis, peritonitis, etc [28]. Our experimental validation using *Staphylococcus hominis* in a mouse model of pancreatitis confirmed its role in

exacerbating RAP, evidenced by increased pancreatic inflammation, higher levels of serum amylase and pro-inflammatory cytokines and elevated macrophage infiltration. This highlights the importance of considering microbial factors in the management and prevention of recurrent pancreatitis.

While our study provides valuable insights, there are several limitations to consider. First, the sample size was relatively small, which may limit the generalizability of our findings. Larger cohort studies are needed to validate these results. Second, our study design was cross-sectional, making it challenging to establish causality between the identified microbial signatures and RAP. Longitudinal studies would be beneficial to assess the temporal relationship between microbial changes and pancreatitis recurrence. In addition, while we identified exacerbation of RAP in *Staphylococcus hominis*-infected mice, the underlying mechanisms by which this bacterium influences pancreatitis remain unclear. Future studies should focus on elucidating these mechanisms through in-depth functional analyses and experimental models.

CONCLUSION

In conclusion, our study elucidates the distinct microbial and clinical features associated with RAP, emphasizing the role of specific bacterial taxa in the recurrence of pancreatitis. The identification of microbial biomarkers and their functional implications offers new avenues for research and clinical intervention. Future studies should focus on exploring the causal relationships between these microbial communities and pancreatitis recurrence, potentially leading to innovative therapeutic approaches aimed at modulating the gut microbiome to prevent RAP. Additionally, the integration of microbial profiling into clinical practice could enhance the precision of prediction of RAP and the effectiveness of personalized treatment strategies for patients with RAP.

AUTHORS CONTRIBUTIONS

Qiwen Wang, Haorui Zheng and Zengkan Du conceived and designed the study. Qiwen Wang, Haorui Zheng and Zengkan Du carried out experiments. Xinyao Chang and Zining Hang conducted data analysis. Qiwen Wang, Xinyao Chang and Zining Hang collected clinical samples. Qiwen Wang and Zhuan Liao wrote the manuscript. All authors read and approved the final manuscript.

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DATA AVAILABILITY

Data is provided within the manuscript or supplementary information files.

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