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Gut microbiome and metabolite signatures for predicting acute kidney transplant rejection: a prospective study

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Acute rejection (AR) remains a significant challenge in kidney transplantation (KT) despite advances in immunosuppressive treatment. Recognizing the critical influence of the gut microbiome on modulating host immunity, we investigated the association between gut dysbiosis and AR in KT recipients. A total of 97 patients with KT were prospectively enrolled from two centers, and their samples were collected at multiple time points, such as pre-transplant (*n* = 97), three months (*n* = 66), and twelve months (*n* = 37) post-transplant. Microbial profiling was performed using 16S rRNA sequencing and fecal metabolomics was done via nuclear magnetic resonance spectroscopy. Thirty-three patients developed AR after KT, exhibiting reduced bacterial richness and diversity compared with KT recipients without AR. In addition, these patients had increased *Escherichia–Shigella* and decreased *Phascolarctobacterium* abundance. Pathway analysis identified 47 enriched pathways in AR patients, notably those involved in lipopolysaccharide biosynthesis and short-chain fatty acid metabolism. Consistent results were obtained from stool metabolomics, showing reduced propionate and lactate concentrations compared with patients without AR. Finally, combining pre-KT bacterial and fecal metabolite features with clinical parameters significantly improved AR prediction accuracy. Our results suggest that integrating clinical, microbial, and metabolomic data may provide a more holistic patient care regimen across both pre- and post-transplant phases.

Keywords Acute rejection, Kidney transplantation, Gut microbiome

Kidney transplantation (KT) is the treatment of choice for enhancing the survival and quality of life of patients with end-stage kidney disease (ESKD)[1,2]. Despite advancements in immunosuppressive therapies, immune monitoring, and infection prevention strategies, acute rejection (AR) remains a significant challenge, occurring in 10–30% of post-KT patients within the first year[3,4]. Early AR is linked to reduced graft survival[5,6] and increased mortality due to the intensive use of immunosuppressive medications, which can lead to infections, cancer, and cardiovascular diseases[6]. Identifying patients at high risk of AR for early diagnosis and management is crucial. Although several pre-transplant risk factors have been identified[5,7–11], their predictive power is incomplete,

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and many are non-modifiable. This highlights the urgent need for novel, specific, controllable, and noninvasive predictive tools for AR development.

Over the past two decades, it has become evident that symbiotic microbiota significantly influence host immunity and metabolism[12,13]. The gut microbiota of KT recipients is particularly susceptible to alterations that can contribute to complications, including AR. First, individuals awaiting KT often suffer from uremia, a condition associated with gut dysbiosis, due to advanced chronic kidney disease[14,15]. Second, the common underlying conditions in these patients, such as diabetes mellitus and hypertension, are cardiometabolic diseases known to be associated with dysbiosis[16,17]. Third, an array of medications, including immunosuppressants and antibiotics administered to KT recipients, can affect the gut microbiota[18]. Conversely, gut dysbiosis has been linked to post-KT complications, such as diarrhea[19], infection[20], and post-transplant diabetes mellitus[21], all of which may influence the level of immunosuppressive treatment required.

Beyond compositional alterations, gut-derived metabolites provide mechanistic links between the microbiome and transplant outcomes, while representing promising non-invasive biomarker candidates for AR risk stratification. Short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, facilitate regulatory T cell (Treg) induction and promote allograft tolerance[22], with diminished SCFA production capacity associated with rejection episodes[23]. In contrast, microbiota-derived uremic toxins, such as indoxyl sulfate and p-cresyl sulfate, along with trimethylamine-N-oxide (TMAO), may persist at elevated levels following transplantation and are correlated with graft dysfunction[24,25]. Although several studies have reported associations between certain gut microbiota profiles[26–28] or gut-derived metabolites[22] and AR or allograft immune tolerance, these studies were limited by small sample sizes, cross-sectional or retrospective designs, and single-time-point post-KT sampling. To address these limitations, we conducted a prospective cohort study to identify metagenomic biomarkers that can predict AR in KT recipients.

Materials and methods

Patient cohort of KT

Adult patients who underwent KT between August 2016 and March 2019 at two tertiary medical centers in South Korea were prospectively recruited, excluding those who had undergone retransplantation or multi-organ transplantation. Induction therapy was administered according to the immunologic risk stratification. Standard-risk recipients received basiliximab 20 mg intravenously on postoperative days 0 and 4. High-risk recipients received rabbit anti-thymocyte globulin at 1.5 mg/kg/day for 3–4 consecutive days, with the first dose administered intraoperatively. Maintenance immunosuppression consisted of a triple-drug regimen including tacrolimus, mycophenolate mofetil, and corticosteroids. Corticosteroid therapy consisted of intraoperative methylprednisolone 500 mg, followed by conversion to oral prednisolone with gradual tapering to a maintenance dose of 5 mg daily. The desensitization protocol was tailored according to the patient's degree of sensitization, involving either rituximab monotherapy or a combination of plasmapheresis and rituximab therapy. Rituximab (375 mg/m^2) was administered as a single dose 2–3 weeks prior to KT. Intravenous immunoglobulin (IVIG) was administered at a dose of 0.1–0.2 g/kg following each plasmapheresis session[29].

At our institutions, KT recipients undergo protocol allograft biopsies at 10 days and 1 year postoperatively, except when patients are not suitable for percutaneous kidney biopsies. In addition to protocol biopsies, indication biopsies were considered in cases of proteinuria exceeding 1 g/g, a rapidly declining estimated glomerular filtration rate (eGFR), or newly detected anti-HLA donor-specific antibodies, based on the physician's decision. The histopathological diagnosis of renal allograft biopsy was performed according to the 2017 Banff classification[30]. Recipients were divided into two groups: the AR group experienced any episode of AR, including antibody-mediated rejection (AMR) or T cell-mediated rejection (TCMR), within the first year post-transplantation, whereas the non-rejection group did not. Patients diagnosed with borderline rejection were classified into the non-rejection group, although some received high-dose steroid treatment. We collected diverse clinical data by reviewing electronic medical records.

Fecal samples collection and 16S rRNA amplicon sequencing

Stool samples were collected in a series at pre-transplant, and then at 3 and 12 months post-transplant, using the methods established in our prior research[31]. DNA extraction was conducted with the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions[32]. We amplified the 16S rRNA gene's V4-V5 region for the sequencing library, which was then sequenced using the Illumina MiSeq system (Illumina, San Diego, CA, USA), as previously detailed[31].

This study was approved by the Institutional Review Board (IRB) of two centers (Seoul National University Hospital, IRB No. 1703-062-839; and Seoul Metropolitan Government Seoul National University Boramae Medical Center, IRB No. 06-2011-50) and adhered to the Declaration of Istanbul and Helsinki principles. Clinical data and fecal samples were prospectively collected with informed consent from the study patients.

Bioinformatics analyses

The 16S rRNA sequencing data were processed using QIIME2 (version 2020.8)[33], with DADA2[34] employed for denoising amplicon sequence variants (ASVs), and taxonomic classification performed using the SILVA 138 operational taxonomic unit (OUT) reference database[35,36]. Alpha diversity metrics, including observed ASVs and the Shannon index, were analyzed using Wilcoxon rank-sum and Kruskal–Wallis tests, with false discovery rate (FDR) correction for significance. Beta diversity was assessed using R's vegan package (v2.5-7). ALDEx2[37] and MaAsLin[38] in R were used to identify differentially abundant taxa and their association with clinical variables. To specifically identify microbial taxa associated with AR while accounting for potential clinical confounders, our MaAsLin analysis employed a multivariable model. In this model, the relative abundance of each genus was modeled as a function of the primary variable of interest (AR status), with adjustments for a comprehensive

set of pre-transplant clinical and demographic covariates, including age, sex, BMI, diabetes mellitus status, perioperative antibiotic use, use of immunosuppressive agents, and desensitization therapy as fixed effects. For these analyses, we considered FDR-corrected q-values below 0.25 to be significant. This approach is a standard practice in exploratory microbiome discovery studies, where a more permissive FDR is adopted to account for the inherently high variability and data sparsity. This strategy mitigates the high rate of false negatives that can result from overly conservative thresholds, allowing for a more comprehensive identification of potentially significant microbial associations that warrant further investigation[39–41].

Gut microbiome functions inferred from the 16S rRNA data via PICRUSt2[42] were analyzed for pathway abundance differences using LEfSe[43], with significance and effect sizes determined by the Wilcoxon rank-sum test and linear discriminant analysis (LDA), respectively. Comprehensive bioinformatics methods are described in the Supplementary Material.

Statistical analysis

In the comparison of demographic and clinical information between the AR and non-rejection groups, categorical variables are presented as numbers with percentages and compared using either the chi-square test or Fisher's exact test. Continuous data are presented as means (with standard deviation) and analyzed using a two-sample t-test or as medians (with interquartile range) and assessed using the Wilcoxon rank-sum test. Statistical significance was defined as a two-tailed P value < 0.05.

To model the longitudinal changes in the relative abundance of key genera over time between the AR and non-rejection groups, we used multivariate linear mixed-effects (LME) regression models, implemented with the lmer function in the R package lme4 (v1.1-27.1). This analysis utilized all available longitudinal samples from 97 patients. A coefficient of zero for group × time and a nonzero coefficient for group indicated a time-independent effect of the group on genus relative abundance.

Receiver operating characteristic (ROC) analysis, including the calculation of the area under the curve (AUC), was conducted using the *pROC* package in R to assess whether the AR group could be differentiated from the non-rejection group based on bacterial composition, metabolites, and clinical characteristics. The clinical variables included age, sex, BMI, pre-transplant use of immunosuppressive agents and antibiotics, diabetes mellitus, donor relation and type, dialysis modality, ABO incompatibility, HLA mismatch, induction regimen, cause of ESKD, presence of preformed donor-specific antibodies, and use of a desensitization protocol.

Sensitivity analysis for outcome heterogeneity

To address potential biases due to outcome heterogeneity, we performed a series of sensitivity analyses. First, we defined a "strict non-rejection" cohort by excluding all patients with a diagnosis of borderline rejection. This cohort was used as the control group for two subsequent comparisons: (1) against the total AR group to validate our primary findings and (2) in an exploratory analysis against each AR subtype: TCMR, AMR, and mixed rejection. For these comparisons, we re-evaluated alpha diversity (Observed ASVs, Shannon index), beta diversity (Bray-Curtis dissimilarity with PERMANOVA), and performed differential abundance analyses using MaAsLin and ALDEx2 to identify subtype-specific taxonomic signatures.

NMR-based stool metabolomics

Stool metabolite analysis was performed using ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The procedures for metabolite extraction, spectral acquisition, and NMR data processing are described in the Supplementary Material.

Results

Baseline characteristics and outcomes of the KT recipients

During the study period, 302 patients underwent KT, from whom 136 pre-transplant fecal specimens were obtained. Out of these, a total of 200 samples from 97 patients with complete clinical data were included in the final analysis: 97 pre-transplant, 66 at three months post-transplant, and 37 at twelve months post-transplant (Fig. 1).

Pre-transplant samples were used to identify baseline signatures for AR prediction, while post-transplant samples served to describe longitudinal changes. The cohort had a mean age of 49.9 ± 12.0 years, and 60.8% were male. Most patients (83.5%) received living-donor kidneys, and 32.0% underwent desensitization. Induction with anti-thymocyte globulin was implemented in 13.5% of the recipients. All patients received standard maintenance immunosuppressive therapy with tacrolimus, mycophenolate mofetil, and steroids.

Within the first year of post-transplantation, 96 of 97 patients (99.0%) underwent allograft biopsy: protocol biopsy alone ($n = 72$) or both protocol and indication biopsies ($n = 25$). One patient received neither protocol nor indication biopsy and, based on stable graft function with no clinical suspicion of rejection, was classified as belonging to the non-rejection group. Among them, 33 individuals (34%) developed AR, with a median time to AR of 9 days (interquartile range, 9–63 days). TCMR was the most common (19, 57.6%), followed by AMR (11, 33.3%), and mixed rejection (3, 9.1%). In comparisons between the AR and non-rejection groups (Table 1), the AR group had a lower number of related live donors, a higher number of HLA mismatches, and received more desensitization treatments. During the same period, several adverse events occurred, including new-onset diabetes mellitus after transplantation ($n = 17$), bacterial infections ($n = 24$), viral infections ($n = 11$), graft failure ($n = 3$), and death ($n = 3$), although there were no significant differences between the AR and non-rejection groups.

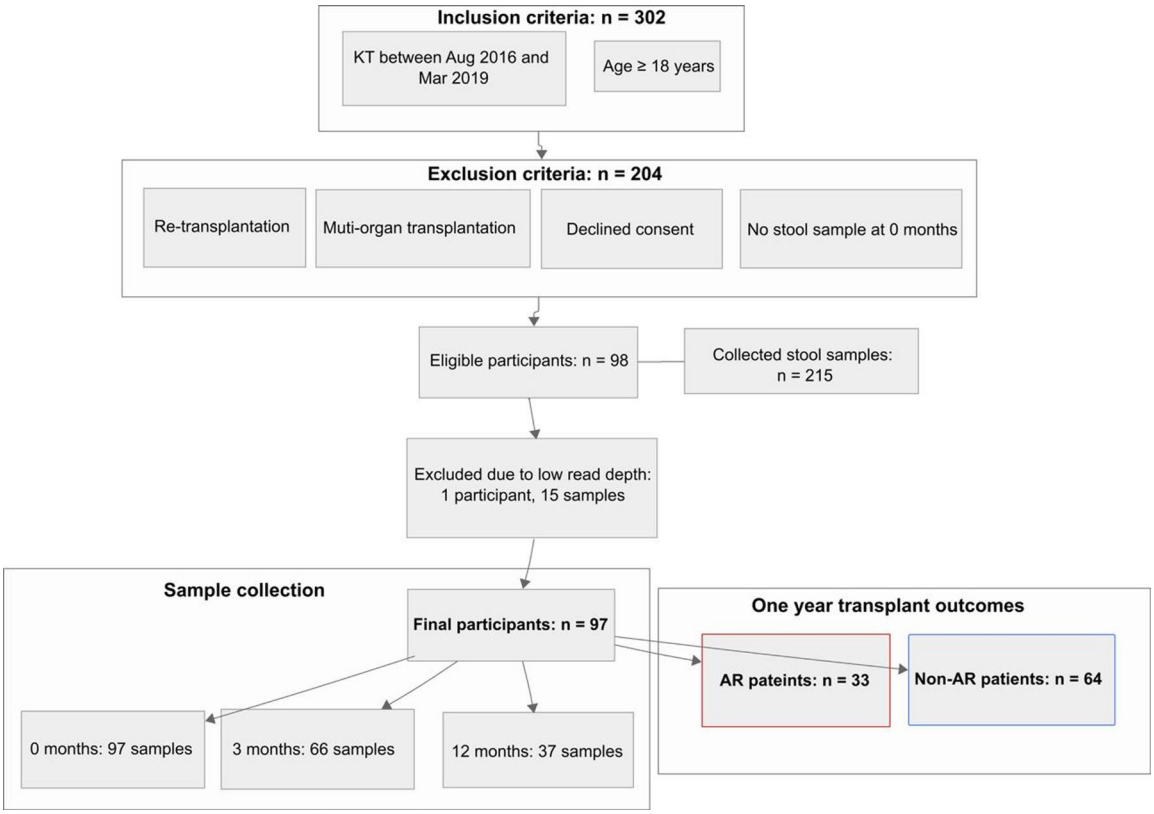


Fig. 1. Flow diagram of fecal sample collection and patient classification. A total of 98 kidney transplant (KT) recipients provided 214 fecal samples collected at three time points: pre-transplantation, 3 months post-transplantation, and 12 months post-transplantation. One recipient and 14 samples were excluded because of low sequencing read depth, resulting in 200 samples included in the analysis. Within the first year after KT, 33 recipients experienced biopsy-proven acute rejection (AR group), whereas the remaining 64 did not (non-rejection group).

Gut Microbiome diversity and composition

Pre-transplant bacterial richness and diversity were significantly lower in the AR group compared with the non-rejection group (P_{FDR} = 0.07 and P_{FDR} = 0.03, respectively; Fig. 2A). Beta diversity analyses demonstrated distinct microbial community structures between the groups (PERMANOVA, P = 0.003; Fig. 2B).

These alpha and beta diversity differences persisted for three months after transplantation (Supplementary Fig. 1A and 1B), but dissipated by the 12-month post-KT (Supplementary Fig. 1C and 1D). Among clinical factors, antibiotic exposure within 90 days before sampling was marginally associated with reduced gut microbial diversity (P_{FDR} = 0.056), while sex (PERMANOVA P = 0.029) and desensitization treatment (PERMANOVA P = 0.019) significantly influenced gut microbial composition (Supplementary Fig. 2). When we explored the bacterial community at the genus level, *Bacteroidetes* was the predominant genus in both the AR and non-rejection groups (Fig. 2C).

Taxonomic differences between AR and non-rejection groups

Pre-transplant differential abundance analyses identified a higher *Escherichia-Shigella* abundance (q < 0.25; Fig. 3A and Supplementary Table 1) and lower *Phascolarctobacterium* abundance (q < 0.25; Fig. 3B and Supplementary Table 1) in the AR group.

The same analysis at the ASV level confirmed these results (Supplementary Table 2). After adjusting for clinical covariates, only a lower level of *Phascolarctobacterium* (q < 0.25; Fig. 3C) was significantly associated with AR. Conversely, the relative abundance of *Escherichia-Shigella* did not differ significantly between the AR and non-rejection groups (q = 0.54).

Longitudinal analysis using all available data from the full cohort confirmed persistent lower *Phascolarctobacterium* (β = −0.0265; 95% CI, − 0.0488 to − 0.0042) and higher *Escherichia-Shigella* (β = 0.1547; 95% CI, 0.0131–0.2963) in AR patients over time (Fig. 4, Supplementary Table 3), with an overall increase in *Escherichia-Shigella* regardless of rejection status (β = 0.0546; 95% CI, 0.0165–0.0926). However, in a sensitivity analysis exclusively of the 37 patients with complete follow-up (Supplementary Table 4), the association of *Escherichia-Shigella* with AR lost significance, whereas *Phascolarctobacterium* depletion remained robust (β = -0.0358; 95% CI, -0.0709 to -0.0007). The significant increase of *Escherichia-Shigella* over time remained consistent in the completer cohort (β = 0.0587; 95% CI, 0.0143 to 0.1032) (Supplementary Table 3).

	Non-rejection (*N* = 64)	Acute rejection (*N* = 33)	*P*-value
Demographics and anthropometrics			
Age, years	50.4 ± 11.9	48.9 ± 12.5	0.577
Male	36 (56.2)	23 (69.7)	0.286
BMI, kg/m^2	22.6 ± 3.5	23.6 ± 4.1	0.255
Hospital			
SMG-SNU	13 (20.3)	3 (9.1)	0.262
SNUH	51 (79.7)	30 (90.9)	
Diabetes Mellitus	16 (25.0)	11 (33.3)	0.530
Hypertension	46 (71.9)	21 (63.6)	0.549
Cause of ESKD			
Diabetes Mellitus	13 (20.3)	9 (27.3)	0.466
Glomerulonephritis	21 (32.8)	5 (15.2)	
Hypertension	2 (3.1)	1 (3.0)	
Polycystic kidney disease	8 (12.5)	3 (9.1)	
Others	3 (4.7)	3 (9.1)	
Unknown	17 (26.6)	12 (36.4)	
Modality			
Preemptive	27 (42.2)	9 (27.3)	0.389
Hemodialysis	29 (45.3)	21 (63.6)	
Peritoneal dialysis	6 (9.4)	2 (6.1)	
Hemodialysis + Peritoneal dialysis	2 (3.1)	1 (3.0)	
Dialysis duration, months	19.3 ± 33.5	25.0 ± 34.0	0.432
Donor type			0.048
Deceased donor	12 (18.8)	4 (12.1)	
Living donor	52 (81.3)	29 (87.8)	0.030
Related	29 (55.8)	8 (27.9)	
Spouse	21 (40.4)	17 (51.6)	
Others	2 (3.8)	4 (12.1)	
ABO incompatibility	12 (18.8)	12 (36.4)	0.098
HLA mismatch			
>3	26 (40.6)	22 (66.7)	0.027
≤3	38 (59.4)	11 (33.3)	
Preformed DSA	10 (15.6)	9 (27.3)	0.272
Perioperative medication			
Antibiotics	14 (21.9)	6 (18.2)	0.872
Antiviral agent	5 (7.8)	3 (9.1)	1.000
Desensitization	15 (23.4)	16 (48.5)	0.023
IVIG + Plasmapheresis	12 (18.8)	9 (27.3)	0.481
Immunosuppressive agent	16 (25.0)	11 (33.3)	0.530
Induction agent			
Anti-thymocyte globulin	7 (10.9)	6 (18.2)	0.320
Basiliximab	56 (87.5)	26 (78.8)	
Both	0 (0.0)	1 (3.0)	
None	1 (1.6)	0 (0.0)	
Complications after KT			
PTDM	11 (17.2)	6 (18.2)	1.000
Continued			

	Non-rejection ($N=64$)	Acute rejection ($N=33$)	P-value
Bacterial infection	12 (19.4)	12 (37.5)	0.096
Viral infection	6 (9.4)	5 (15.2)	0.609
Delayed graft function	0 (0.0)	1 (3.0)	0.735
Graft failure	0 (0.0)	3 (9.1)	0.067
Death	0 (0.0)	3 (9.1)	0.067

Table 1. Comparisons of baseline characteristics between the acute rejection and non-rejection groups. Values are expressed as means ± standard deviation, or n (%). BMI, body mass index; SMG-SNU, Seoul Metropolitan Government Seoul National University Boramae Medical Center; SNUH, Seoul National University Hospital; ESKD, end-stage kidney disease; HLA, human leukocyte antigen; DSA, donor-specific antibody; IVIG, intravenous immunoglobulin; KT, kidney transplantation; PTDM, posttransplant diabetes mellitus.

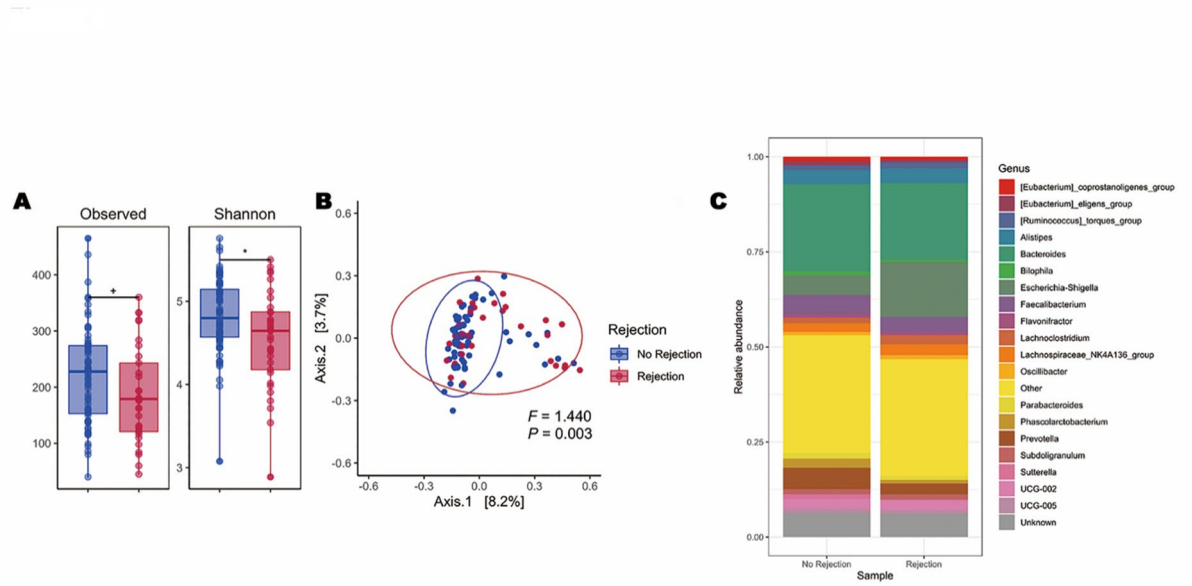


Fig. 2. Alpha and beta diversity and gut microbiota composition before kidney transplantation in recipients with and without acute rejection. (**A**) Comparison of alpha diversity between the acute rejection (AR) and non-rejection groups, evaluated by the number of observed amplicon sequence variants (ASVs) and Shannon diversity index. (**B**) Principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarity showing clustering of gut microbial communities by rejection status. (**C**) Relative abundance of gut microbiota at the genus level in the AR and non-rejection groups prior to transplantation. $^{*}P<0.05$; $^{+}P<0.1$.

Microbial diversity and signatures of rejection subtypes

Sensitivity analysis excluding borderline rejection cases confirmed our primary findings. Microbial diversity was lower in the AR ($n=33$) than in the strict non-rejection group ($n=43$) (Shannon index, P_{FDR} = 0.07; Supplementary Fig. 3A), and the microbial community structure remained distinct (PERMANOVA, $P=0.02$; Supplementary Fig. 3B). Distinct microbial community profiles were observed among the TCMR, AMR, and mixed rejection groups (PERMANOVA, $P=0.013$; Supplementary Fig. 4B), with lower diversity in the mixed rejection group (Observed ASVs, $P_{FDR}=0.033$). Although not reaching statistical significance, a consistent trend of decreasing diversity was also observed in the AMR group ($P_{FDR}=0.36$), as well as in both the mixed and AMR groups based on the Shannon diversity index (P_{FDR} = 0.053 and P_{FDR} = 0.078, respectively) (Supplementary Fig. 4A).

Furthermore, differential abundance analyses uncovered unique taxonomic signatures. The depletion of *Phascolarctobacterium* was primarily driven by the TCMR subgroup (MaAsLin, $q=0.186$), whereas the mixed rejection group was characterized by a significant enrichment of the family *Enterobacteriaceae* and the genus *Sutterella* (ALDEx2, Welch's t-test corrected $q<0.05$). No significant taxa were identified in the AMR group, likely due to the limited statistical power of the small subgroup size (Supplementary Fig. 4C and Supplementary Table 5).

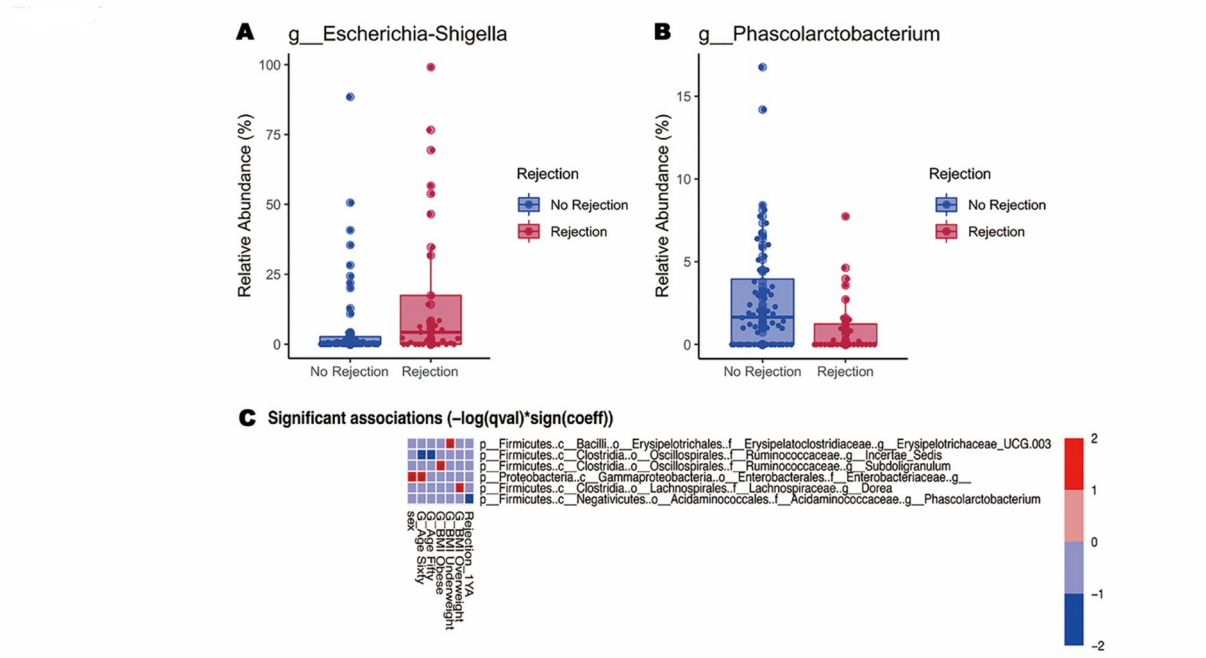


Fig. 3. Specific taxa associated with acute rejection. Box plots depict the significant associations of acute rejection (AR) with (**A**) the genus *Escherichia-Shigella* and (**B**) the genus *Phascolarctobacterium*. The ALDEx2 analysis was employed to identify different taxa, and only two genera were identified with wi.eBH < 0.25. (**C**) Association of *Phascolarctobacterium* with AR status based on multivariable analysis using MaAsLin, with a significance threshold of $q < 0.25$.

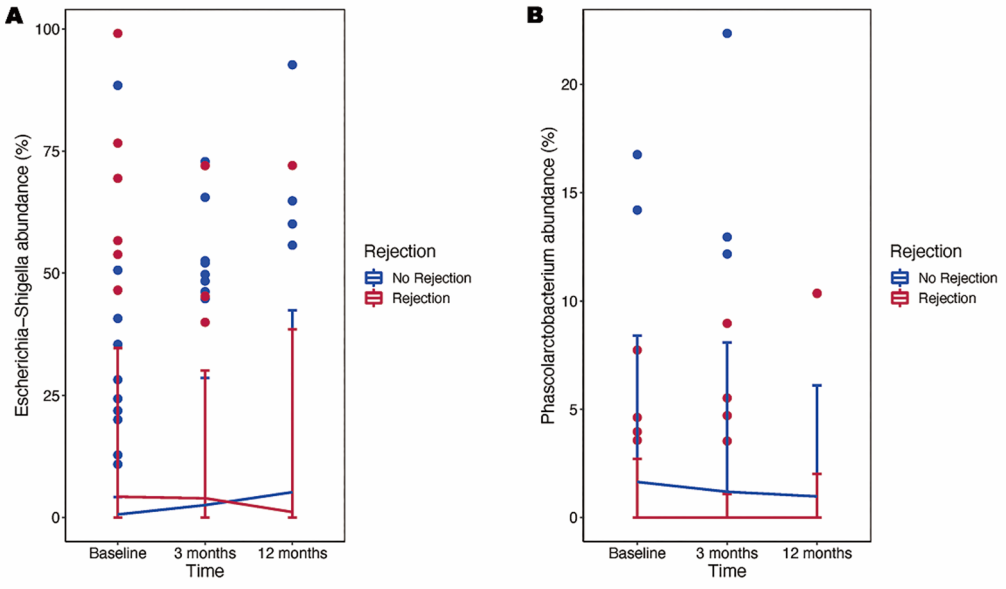


Fig. 4. Longitudinal changes in the relative abundance of key taxa in relation to the transplant period and acute rejection. The relative abundance of *Escherichia-Shigella* (**A**) and *Phascolarctobacterium* (**B**) was modeled over time using a linear mixed-effects (LME) model adjusted for age, sex, and body mass index. Analyses were performed to assess the interaction between the transplant period and acute rejection status.

Functional pathways associated with AR

PICRUSt2 and LEfSe analyses identified 77 pathways enriched between groups (LDA score > 2.5 and $P < 0.05$; Fig. 5 and Supplementary Table 6).

Among the 47 pathways enriched in the AR group, three (PWY0-1338, PWY-7315, and ECASYN-PWY) were involved in lipopolysaccharide (LPS) biosynthesis. Notably, four pathways (P461-PWY, FERMENTATION-PWY, P161-PWY, and ANAEROFRUCAT-PWY) were responsible for the generation or degradation of SCFAs (precursor metabolites were converted to metabolic byproducts such as lactate and acetate), and FAO-PWY was responsible for the degradation of fatty acids and lipids. Furthermore, two pathways (PWY-5747 and PWY0-42) were involved in the degradation of propionate, a key SCFA. The biosynthesis of colanic acid building blocks, specifically attributed to COLANSYN-PWY, releases pro-inflammatory molecules[44]. Compared with the AR group, three pathways enriched in the non-rejection group (PEPTIDOGLYCANSYN-PWY, PWY-6385, and PWY-6387) were involved in peptidoglycan biosynthesis.

Stool metabolite differences between the AR group and non-rejection group

Next, to identify stool metabolites reflecting underlying bacterial functional pathways, we analyzed the water-soluble fecal metabolome of 75 participants (27 with AR and 48 without AR) using ^{1}H NMR spectroscopy. Each metabolite feature was plotted on a volcano plot, displaying the fold change in normalized areas between the two groups against their statistical significance (Fig. 6A). Of the 164 metabolite features analyzed, none remained statistically significant after FDR correction for multiple tests ($P_{FDR} > 0.05$). However, the AR group showed reduced fecal concentrations of lactate (15.2% reduction; 95% CI: 2.1–28.2%; $P_{raw} = 0.024$), with a chemical shift at 1.3437 ppm, and propionate (13.7% reduction; 95% CI: 0.6–26.7%; $P_{raw} = 0.040$) at 1.0574 ppm, compared with the non-rejection group (Fig. 6B).

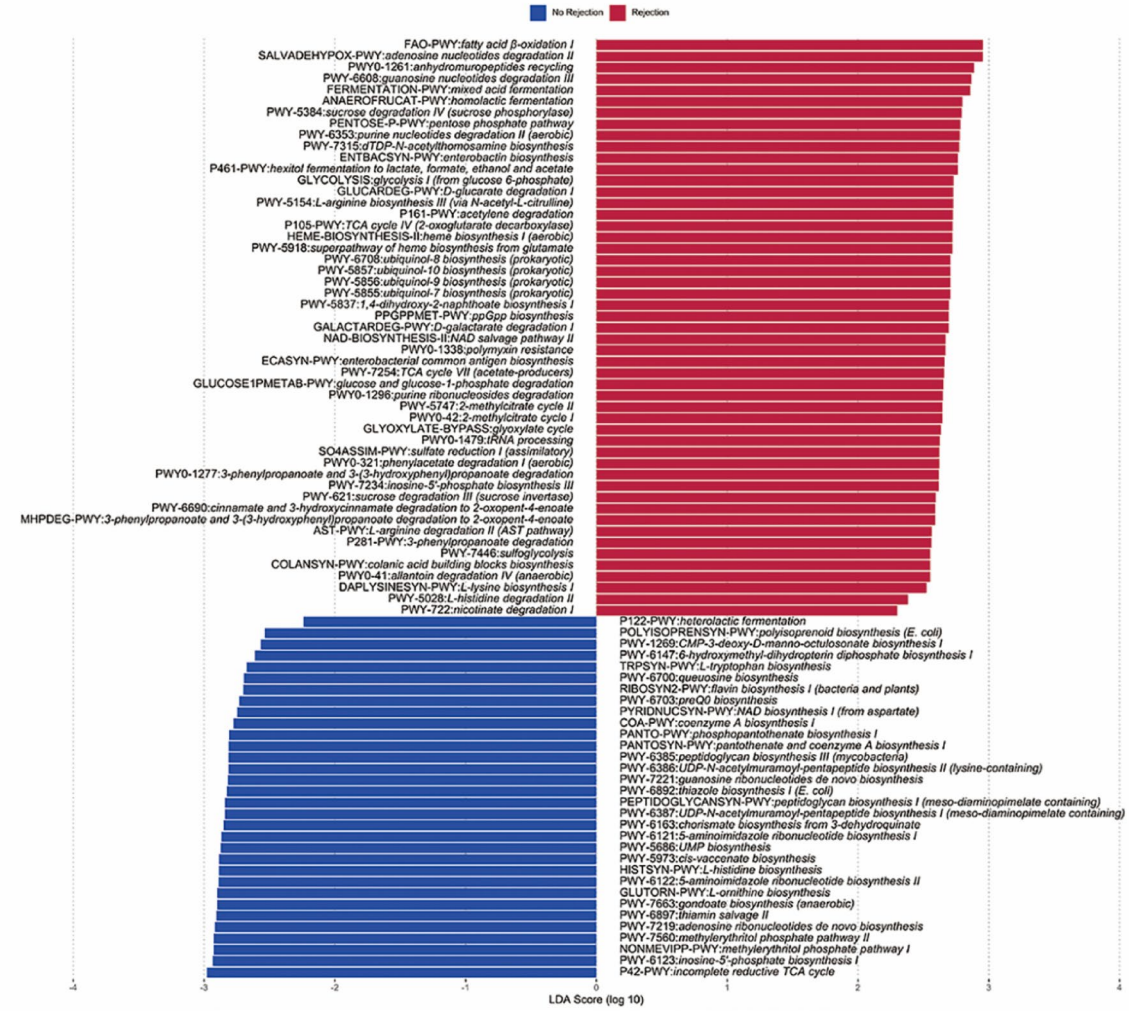


Fig. 5. Predicted microbial metabolic pathways associated with acute rejection. Differentially abundant functional pathways between the acute rejection (AR) and non-rejection groups were identified using LEfSe analysis (LDA score > 2.5, $P < 0.05$). Predicted pathways were inferred from amplicon sequence variants using the PICRUSt2 pipeline and annotated using the MetaCyc database.

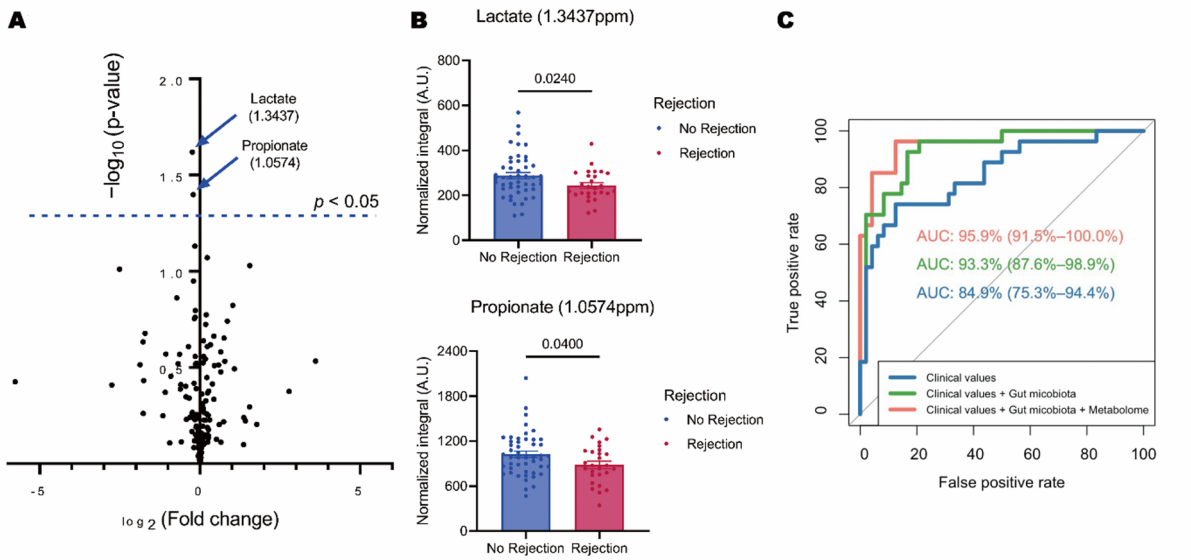


Fig. 6. Stool metabolomic profiling and predictive modeling of acute rejection. (**A**) Volcano plot derived from ^{1}H NMR analysis of the water-soluble fecal metabolome. Metabolites above the horizontal threshold line ($P < 0.05$) were considered significantly different between the acute rejection (AR) and non-rejection groups. (**B**) Lactate (1.3437 ppm) and propionate (1.0574 ppm) levels were significantly lower in the AR group. Box plots illustrate the distribution of each metabolite in each sample, with mean and standard error, and corresponding *P* values from t-test. (**C**) Receiver operating characteristic (ROC) curves evaluating the predictive performance of AR. Models were constructed using clinical variables only (blue), clinical + taxonomy (green), and clinical + taxonomy + metabolites (red). AUC, area under the curve.

Gut microbiome-based AR prediction

Based on these results, we evaluated whether bacterial markers and their associated fecal metabolites could enhance the predicted probability of AR after KT. Adding pre-transplant relative abundances of two bacterial genera (*Escherichia-Shigella* and *Phascolarctobacterium*) to the 13 clinical parameters achieved a higher AUC of 0.933 (95% CI: 0.876–0.989) compared with the clinical value-only prediction of AR with an AUC of 0.849 (95% CI: 0.753–0.944) (Fig. 6C). The addition of two stool metabolites (lactate and propionate) to the clinical/bacterial genera-based model further enhanced AR predictability, although not significantly (AUC: 0.959; 95% CI: 0.915–1.000).

Discussion

Symbiotic gut microbiota may play an important role in the development of AR after KT, although evidence from clinical settings remains limited. In this study, we comprehensively characterized the sequential gut microbial profiles of patients experiencing AR after KT and compared them with those of patients without AR. Our study demonstrated that patients with AR after KT had lower microbial diversity even during the pre-transplant period. Moreover, two bacterial strains, *Escherichia-Shigella* and *Phascolactobacterium*, along with their associated metabolites, including lactate and propionate, were associated with AR after KT. They also contribute to AR prediction when added to clinical parameter-based prediction models. Considering the unmet need for AR prediction in KT recipients, our findings suggest a novel biomarker for AR.

We characterized the dynamics of the gut microbiome from ESKD to KT using sequential samples. Recent studies have demonstrated that lower microbial diversity in transplant recipients is associated with adverse outcomes, including mortality and graft rejection[45,46]. Notably, Holle et al. found that gut microbiome alterations, including decreased microbial diversity, precede graft rejection in kidney transplantation patients, with these changes occurring before the rejection event[23]. However, our study extends these findings by specifically examining longitudinal changes in bacterial diversity from the pre-transplant period and its predictive value for AR development. Pre-KT, the lower bacterial diversity observed in patients with ESKD may reflect the severity of uremia, creating an acidic intestinal environment that could limit the growth of certain types of bacteria intolerant to lower pH levels[47]. In addition, other combined dietary factors, environmental changes, and comorbidities, such as diabetes and obesity, may affect the lower bacterial diversity in patients with ESKD awaiting KT. Interestingly, our results showed a significant difference in bacterial diversity among patients with ESKD. Moreover, lower bacterial diversity in patients with ESKD may affect the occurrence of AR after KT. This finding may be helpful in stratifying the risk of rejection at the time of transplantation and modulating

immunosuppressive treatment after transplantation. Lower bacterial diversity may be associated with altered immune function due to a decrease in certain types of gut bacteria, which can lead to a decrease in the production of SCFAs or an increase in the number of harmful bacteria[48]. Considering the complex mechanisms related to lower bacterial diversity, further comprehensive studies are required to establish causality.

Phascolarctobacterium produces propionate, an SCFA known to support immune homeostasis and anti-inflammatory responses[49,50]. SCFAs are primarily generated through the fermentation of dietary fibers by colonic bacteria. However, this process is often compromised in patients with ESKD due to reduced dietary fiber intake. The resulting decline in SCFA-producing microbes, along with a shift toward uremic toxin-producing species, contributes to systemic inflammation and impaired immune function[51]. Experimental studies have shown that SCFAs attenuate kidney ischemia-reperfusion injury[52]. Furthermore, a high-fiber diet prolongs allograft survival and reduces rejection events by inducing Tregs via SCFA[22]. Consistent with these findings, our metabolomic analysis revealed reduced levels of both propionate and lactate in pre-transplant stool samples from patients with AR. These changes were accompanied by increased activity of microbial pathways related to SCFA fermentation and *Escherichia coli*-mediated propionate degradation (e.g., 2-methylcitrate cycle I and II)[53]. Lactate, a common short-chain hydroxy fatty acid in the intestinal lumen, can be converted to other SCFAs via lactate-fermenting bacteria. Recent research has indicated that lactate and propionate modulate the proinflammatory activation of epithelial and myeloid cells[54]. Overall, these findings suggest that gut-derived lactate and propionate play crucial roles in regulating inflammation and the immune response, ultimately determining the outcome of a kidney allograft, whether it leads to tolerance or rejection.

Conversely, members of the genus *Escherichia-Shigella*, a subset of the *Enterobacteriaceae* family, have been implicated as potential pathogens in solid organ transplantation. *Enterobacteriaceae* overgrowth has previously been associated with AR in small bowel transplant recipients[55], supporting our finding that *Escherichia-Shigella* enrichment may contribute to AR after KT. In an animal model, expansion of *Escherichia* following ischemia-reperfusion injury led to increased production of complex LPS, impaired gut barrier integrity, and systemic inflammation, exacerbating acute kidney injury[56]. LPS, a major structural component of Gram-negative bacterial cell walls, is a potent activator of toll-like receptors (TLRs), which play a key role in innate immune sensing and the initiation of transplant rejection[57]. LPS-TLR signaling can amplify pro-inflammatory responses and facilitate the development of the adaptive immune response involved in AR. Consistent with this, we observed an enrichment of LPS biosynthesis pathways, such as dTDP-N-acetylthiosamine biosynthesis, enterobacterial common antigen biosynthesis, and polymyxin resistance, in the AR group. Taken together, these findings suggest that pre-transplant expansion of *Escherichia-Shigella* contributes to AR through enhanced LPS production and activation of the innate immune response. When considered alongside the concurrent reduction in *Phascolarctobacterium*, these microbial alterations reflect a state of gut dysbiosis that may play a critical role in shaping transplant outcomes. Further investigation is warranted to elucidate the mechanistic links between gut microbial composition, LPS-mediated signaling, and immune activation in the pathogenesis of graft rejection.

The merits of our study include the large cohort size, prospective study design with longitudinal samples, comprehensive clinical and pathological characteristics, and inclusion of pre-transplant samples. However, this study had several limitations. First, the use of 16S rRNA sequencing has constraints in accurately identifying species-level information and capturing functional gene data. Future investigations will benefit from employing shotgun metagenomic sequencing for a more comprehensive analysis. Second, although diet plays a crucial role in shaping the composition of the intestinal microbiota, we did not evaluate dietary factors in our study. Further research is required to explore the potential impact of pre-transplantation diets on the development of AR through changes in intestinal microflora and metabolite levels. Third, although our findings revealed an association between specific gut microbiota composition and AR, causal relationships were not established. Therefore, animal and in vitro experiments are required to elucidate the underlying mechanisms. Fourth, our metabolomics findings did not reach statistical significance after FDR correction for multiple testing, reflecting the exploratory nature of this analysis and requiring further investigation in expanded cohorts. Finally, a key limitation is that our predictive model was developed and evaluated within a single cohort, without a separate validation process. This approach carries an inherent risk of overfitting; thus, the model performance should be interpreted as preliminary. The clinical utility of these biomarkers requires confirmation through rigorous validation in larger, multicenter studies.

This study revealed that pre-transplant changes in specific bacteria and gut-derived metabolites have the potential to predict AR post-transplantation, suggesting potential modifiable targets at the bacterial and metabolomic levels for AR prevention. Further studies are required to clarify the mechanisms of action of bacteria and their metabolites in AR pathogenesis.

Data availability

The sequences obtained in this study are available through the European Nucleotide Archive (ENA) at the European Bioinformatics Institute (EBI) under Accession Number PRJEB93912.

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Author contributions

S.P. and H.L. conceived the study. H.C., J.E.K., J.I.P., and H.L. designed this study. H.C., J.E.K., H.-E.K., J.I.P., Y.C.K., J.P.L., D.K.K., K.W.J., and Y.S.K. contributed to data acquisition. H.C., J.P., and H.N. contributed to the data analysis. H.C., H.N., B.-S.K., S.P., and H.L. contributed to drafting the manuscript. All authors have read and agreed to the final version of this manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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